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Protein Export According to Schedule: Architecture, Assembly, and Regulation of Type III Secretion Systems from Plant- and Animal-Pathogenic Bacteria

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INTRODUCTION

Higher eukaryotes such as plants, animals, and humans are permanently exposed to the risk of bacterial infections, which often lead to severe and even lethal diseases. Major infectious agents are Gram-negative bacteria, which utilize at least six different protein secretion systems (type I to type VI

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secretion systems) to transport bacterial virulence factors into the surrounding milieu or directly into the host cell. Protein secretion systems from Gram-negative bacteria differ significantly in structure, regulation, and substrate specificity and are summarized in recent review articles (198, 215, 218, 247, 253, 258, 453, 582). Similar systems are employed by Gram-positive bacteria, but they also contain an additional type of protein secretion system, designated type VII, that was identified in mycobacteria (558). Most pathogens use a combination of several protein secretion systems to successfully conquer their respective host organisms. Although the impact of secretion systems on bacterial virulence can vary in different pathogens, an essential role in pathogenicity has often been assigned to the type III secretion (T3S) system, which delivers bacterial proteins, so-called effector proteins, into the cytosol of eukaryotic cells (107, 465, 466, 512). This transkingdom protein transport enables the pathogen to interfere with host cellular pathways for its own benefit.

T3S systems are highly complex nanomachines that consist of more than 20 components. The membrane-spanning core apparatus is associated with an extracellular pilus-like appendage that is assumed to serve as a channel for transport of secreted proteins to the host-pathogen interface. The translocation of effector proteins into eukaryotic cells is probably mediated by a bacterial channel-like translocon that inserts into the host plasma membrane (Fig. 1A). Notably, the term "T3S system" does not refer only to secretion systems that translocate effector proteins (so-called translocation-associated T3S systems) but also to the bacterial flagellum, which is a key motility organelle and is connected via a hook to the bacterial filament (Fig. 1B). In contrast to translocation-associated T3S systems, flagellar T3S systems mainly secrete extracellular components of the flagellum, such as hook and filament proteins. However, the secretion of virulence factors by flagellar T3S systems has also been observed (617, 618).

Given the architecture of T3S systems, it is assumed that T3S is a hierarchical process and that extracellular components of the secretion apparatus are secreted prior to effector proteins. Similarly, the secretion of hook components of flagellar T3S systems probably precedes the secretion of filament proteins. In the past 5 years, significant progress has been made in the analysis of the structures and functions of many core components of T3S systems as well as of T3S-associated control proteins. The aim of this review is to summarize our current knowledge of the architecture of T3S systems and the control mechanisms underlying T3S in plant- and animal-pathogenic bacteria. For a detailed description of individual proteins or regulatory mechanisms, the reader is also referred to excellent previous overview articles that provide summaries on the following topics: translocation-associated T3S systems (29, 72, 105, 161, 199, 217, 557), flagellar T3S systems (92, 161, 343, 377, 428, 549), T3S chaperones (175, 431), structures and functions of individual components of T3S systems (46, 70, 243, 281, 283, 349, 353, 389, 395, 482), and control mechanisms underlying T3S and gene expression (64, 106, 129, 212, 370, 421, 547, 555, 588).

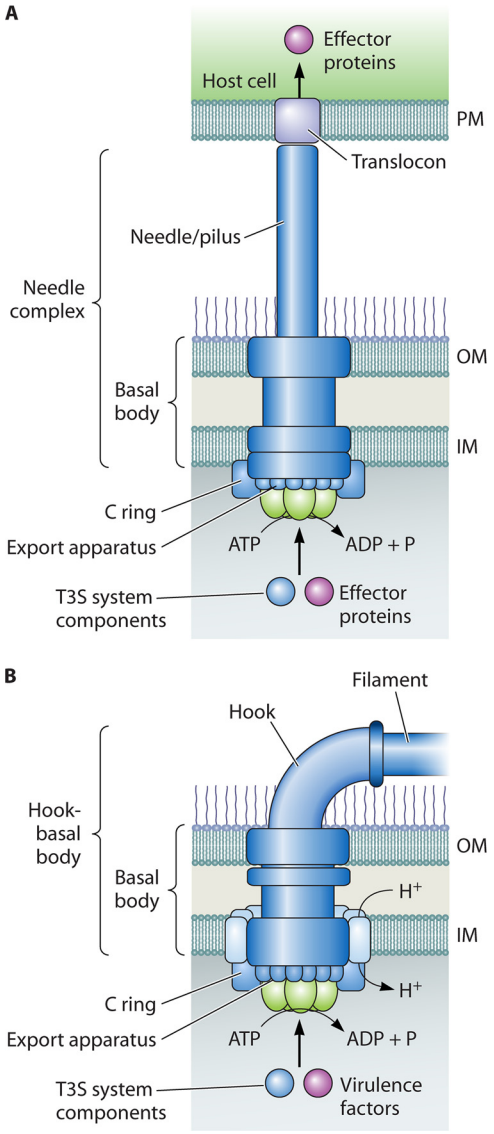
VARIATIONS ON A THEME—DIFFERENCES AND SIMILARITIES OF T3S SYSTEMS

The structural components of T3S systems are encoded by chromosomal or plasmid-borne gene clusters that were probably acquired during evolution by horizontal gene transfer. According to phylogenetic differences in amino acid sequences, T3S systems from animal- and plant-pathogenic or symbiotic bacteria have been classified into different families, including flagellar, Ysc, Inv-Mxi-Spa, Ssa-Esc, Hrp1, and Hrp2 T3S systems as well as T3S systems of the *Chlamydiales* and *Rhizobiales* families (Fig. 1C). Ysc, Inv-Mxi-Spa, and Ssa-Esc-T3S systems have been analyzed intensively in species of the animal-pathogenic bacteria *Yersinia*, *Salmonella*, and *Shigella* and in enteropathogenic *Escherichia coli* (EPEC), while Hrp1 and Hrp2 T3S systems have been studied mainly in the plant-pathogenic bacteria *Xanthomonas* spp., *Ralstonia solanacearum*, and *Pseudomonas syringae*. The Inv-Mxi-Spa T3S system from *Salmonella* spp. and the Ssa-Esc T3S system from EPEC and *Salmonella* spp. are also referred to by the genomic loci that encode them (*Salmonella* pathogenicity island 1 [SPI-1] and SPI-2 for *Salmonella* spp. and locus of enterocyte effacement [LEE] for EPEC) (Fig. 1C).

Many bacteria contain more than one T3S system, including a flagellar T3S system and one or several translocation-associated T3S systems of the same or different families that might be of importance at different stages of the infection process (Fig. 1D). The SPI-1 T3S system of *Salmonella* spp., for instance, promotes bacterial pathogenicity before the invasion of host cells, and the corresponding genes are expressed during the initial bacterial contact with the intestinal epithelium. In contrast, the SPI-2 T3S system is activated only after bacterial entry into the eukaryotic cell cytosol. The different functional requirements of both systems might explain why *Salmonella* spp. possess approximately 10 to 100 SPI-1 T3S systems per cell but only 1 or a few T3S systems of the SPI-2 family type (83, 291). Different translocation-associated T3S systems have also been identified in the animal-pathogenic bacteria *Yersinia* spp. and *Burkholderia* spp. Interestingly, *Burkholderia* spp. contain not only a SPI-1 T3S system but also Hrp-type T3S systems that are usually specific for plant-pathogenic bacteria (449, 522) (Fig. 1D). In most cases, it is still unclear whether the different types of T3S system are required for interactions with different hosts.

Interestingly, translocation-associated T3S systems not only are linked exclusively to bacterial pathogenicity but also can contribute to symbiotic interactions, as shown for the *Rhizobium*-legume symbiosis (173, 574). Genes encoding components of T3S systems have also been identified in other symbiotic (e.g., *Photorhabdus luminescens*, *Sodalis glossinidius*, and the *Sitophilus zeamais* primary endosymbiont) and nonpathogenic bacteria (e.g., *E. coli*, *Pseudomonas fluorescens*, *Desulfovibrio vulgaris*, *Myxococcus xanthus*, and *Verrucomicrobium spinosum*). The precise role of T3S genes during the life cycle of these bacteria remains to be investigated.

Comparative sequence analyses revealed that at least 9 of the more than 20 components of translocation-associated T3S systems are conserved among plant- and animal-pathogenic bacteria. They likely constitute the core components of the secretion apparatus in the inner membrane (IM) and outer membrane (OM). The nomenclature of these proteins refers to the Ysc proteins from the animal-pathogenic bacterium *Yersinia* (48). Eight components are also conserved in the flagellar T3S system, suggesting that the membrane-spanning secretion apparatuses of flagellar and translocation-associated T3S systems share a similar overall architecture. This assumption was confirmed by electron microscopy (EM) studies of isolated fla-



C

Families of translocation-associated T3S systems	Bacterial species that usually contain this T3S system
Ysc	*Yersinia* spp.
Inv-Mxi-Spa	*Salmonella* spp. (encoded by SPI-1) *S. flexneri*
Ssa-Esc	*Salmonella* spp. (encoded by SPI-2) EPEC (encoded by LEE)
Eiv-Epa	*E. coli* (encoded by ETT2)
Hrp1	*P. syringae* *Erwinia* spp.
Hrp2	*Xanthomonas* spp. *R. solanacearum*
Chlamydiales	*Chlamydia* spp.
Rhizobiales	*Rhizobium* spp.

D

Bacterial species	Families of T3S systems
Salmonella spp.	Inv-Mxi-Spa (SPI-1) Ssa-Esc (SPI-2)
Enterohaemorrhagic *E. coli*	Eiv-Epa and Ssa-Esc (LEE)
Yersinia pestis *Yersinia pseudotuberculosis* *Yersinia enterocolitica* serotype 3	Ysc Ssa-Esc
Yersinia enterocolitica biotype 1B	Ysc Inv-Mxi-Spa (Ysa-T3S system)
Burkholderia pseudomallei	2 x Hrp2 Inv-Mxi-Spa
Xanthomonas axonopodis pv. *phaseoli*	Hrp2 Inv-Mxi-Spa
Xanthomonas albilineans	No Hrp-type T3S system Inv-Mxi-Spa (SPI-1)
Vibrio parahaemolyticus	Ysc Hrp2
Burkholderia spp.	Inv-Mxi-Spa 2 x Hrp2

FIG 1 Overview of translocation-associated and flagellar T3S systems from animal- and plant-pathogenic bacteria. (A) Model of the translocation-associated T3S system. The basal body of the T3S system spans the bacterial IM and OM and consists of ring structures that are presumably connected by a periplasmic rod. The basal body is associated via an extracellular needle (animal-pathogenic bacteria) or pilus (plant-pathogenic bacteria) with a channel-like translocon in the host plasma membrane. The basal body and the needle from animal-pathogenic bacteria are referred to as the needle complex. The energy for the docking and unfolding of T3S substrates, including extracellular components of the T3S system and effector proteins, is probably provided by a cytoplasmic ATPase (shown in green) associated with the T3S system. Note that the cytoplasmic C ring is predicted only for translocation-associated T3S systems. A more detailed representation of single components of translocation-associated T3S systems is given in Fig. 2. (B) Model of the flagellar T3S system. The flagellar basal body is associated via an extracellular hook with the flagellar filament, which is 10 to 20 μm long and is the main bacterial motility organelle. The basal body is surrounded by 8 to 11 stator complexes that drive flagellar rotation and contain proton-conducting channels. The flagellar basal body and the hook are referred to as the hook-basal body. A detailed description of individual components of the flagellar T3S systems is provided in Fig. 3. (C) Summary of different families of translocation-associated T3S systems from bacterial pathogens and symbionts of plants or animals. The SPI-1-like Eiv-Epa T3S system encoded by the ETT2 gene cluster from *E. coli* is active in only a few strains. Note that *P. syringae* strains belonging to the phylogenetic subgroup 2c appear to encode an unusual T3S system that is only distantly related to the Hrp1-type T3S system (97). (D) Examples of bacterial species that possess more than one translocation-associated T3S system. Please note that most species contain an additional flagellar T3S system that is not listed in this table.

gellar and translocation-associated T3S systems from *Salmonella* spp. and *Shigella flexneri*, respectively. Both systems consist of ring structures in the IM and OM that enclose a transport channel with an inner diameter of 2 to 3 nm (45, 139, 179, 291, 348, 478) (Fig. 2 and 3). The IM rings are associated with the export apparatus, which is built by members of the YscR, YscS, YscT, YscU, and YscV families and is connected to a predicted cytoplasmic C ring and an ATPase complex (see below). According to a commonly used nomenclature, the IM and OM rings that are linked by a central periplasmic rod structure are

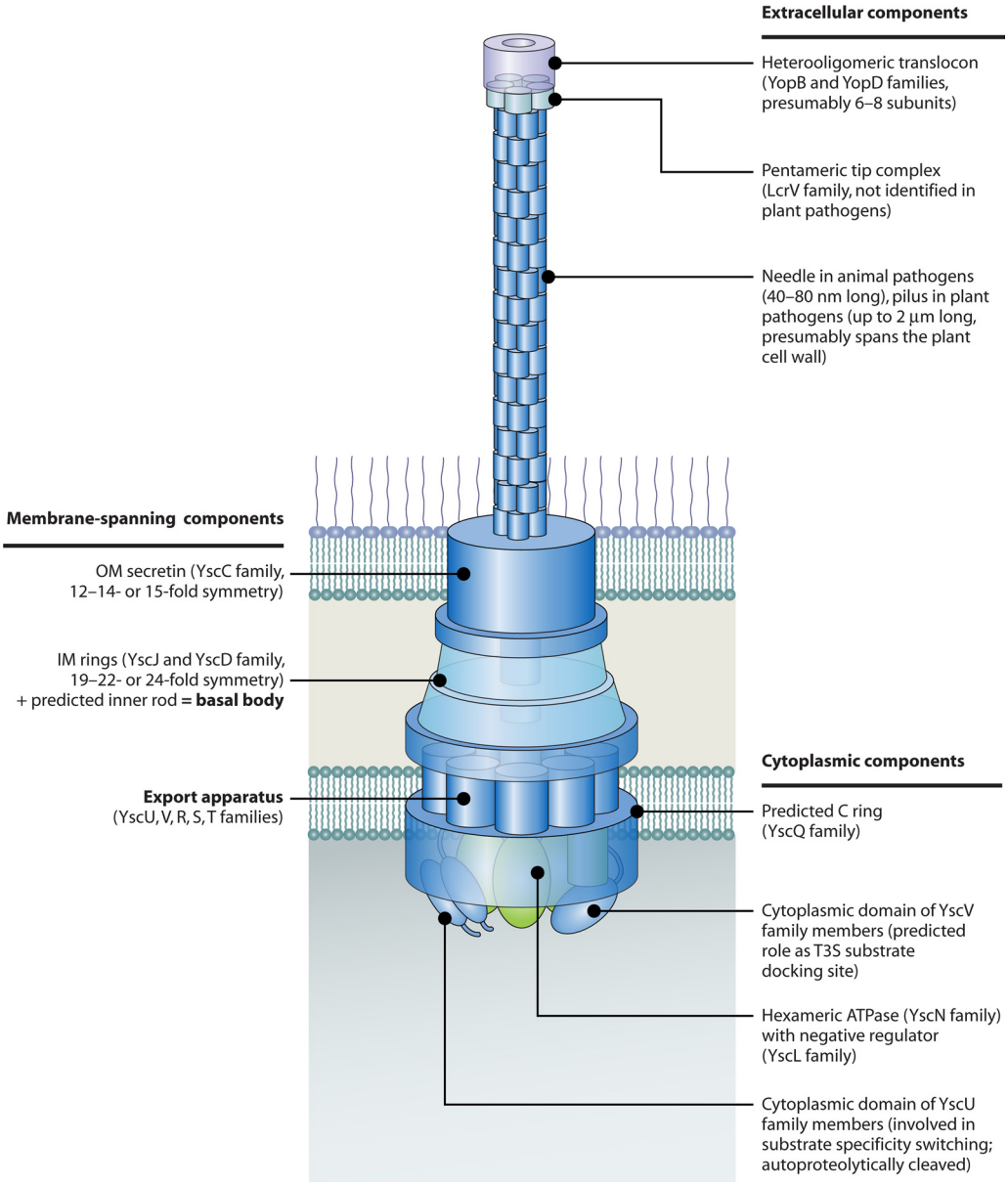


FIG 2 Schematic representation of individual components of translocation-associated T3S systems from animal- and plant-pathogenic bacteria. Conserved membrane-spanning components of the T3S system include the OM secretin (YscC family) and constituents of the IM ring (YscD and YscJ family) and the export apparatus (YscU, -V, -R, -S, and -T families). The IM ring and the export apparatus are associated with the predicted C ring (presumably a multimer of members of the YscQ family) and the hexameric ATPase (depicted in green), which might provide the energy to facilitate the docking and entry of T3S substrates into the inner channel of the secretion system. Additional cytoplasmic components of the T3S system are the predicted regulator of the ATPase (YscL family) and the cytoplasmic domains of YscU and YscV family members, which are probably involved in substrate docking. Note that the composition of the export apparatus and the cytoplasmic parts of the secretion system is speculative and that multiple copies of a single substituent (e.g., members of the YscV protein family) can be involved in the assembly of the T3S system. Extracellular components of the T3S system include the needle (animal-pathogenic bacteria) and pilus (plant-pathogenic bacteria), which differ in length and serve as transport channels for secreted proteins at the host-pathogen interface. Translocation of effector proteins across the host plasma membrane is mediated by the channel-like translocon, which is a hetero-oligomeric protein complex and is connected to the needle via a tip complex that consists of members of the LcrV protein family. Tip complexes have so far been identified and/or characterized only for animal-pathogenic bacteria.

also termed the "basal body." In contrast, the term "needle complex" refers to basal bodies of translocation-associated T3S systems that are associated with the extracellular needle (Fig. 1A). In flagellar T3S systems, the corresponding structure is called the "hook-basal body" (Fig. 1B). Individual components of the needle complex, the hook-basal body, and the export apparatus are discussed in this review and are presented in more detail in Fig. 2 and 3.

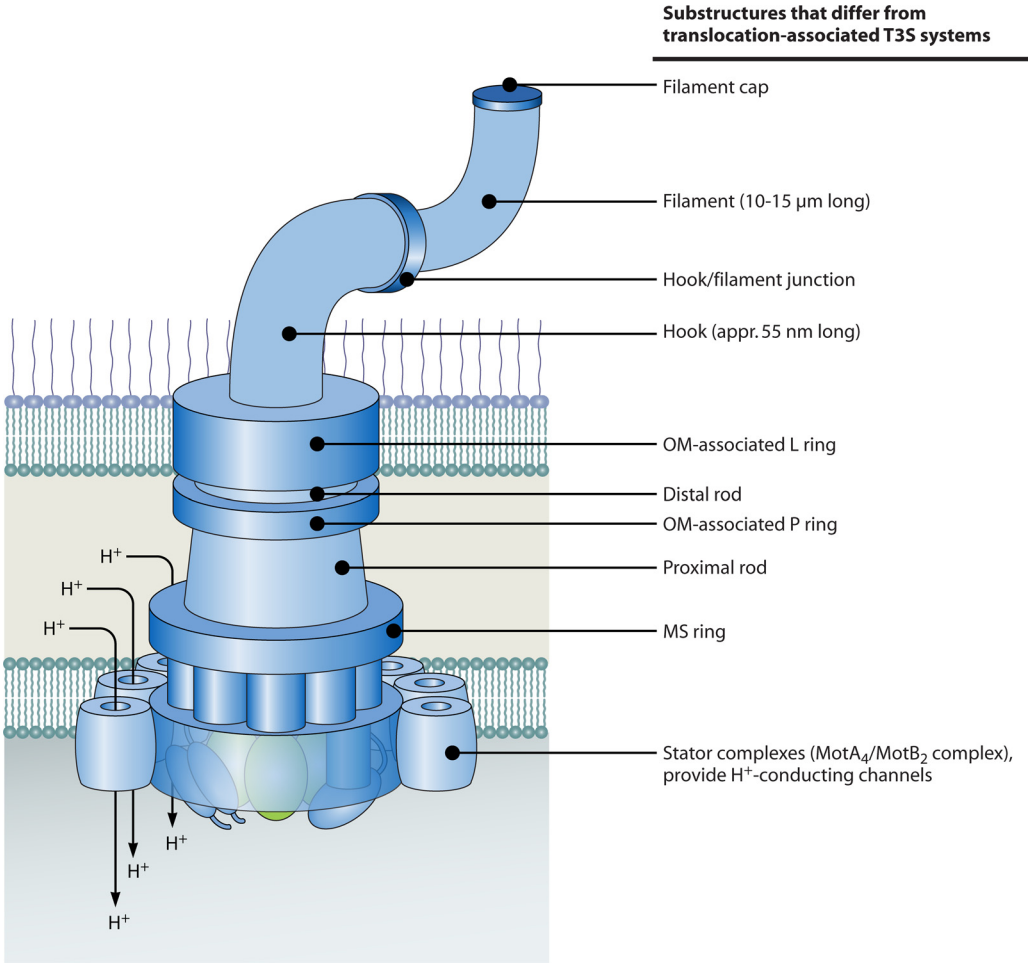


FIG 3 Schematic representation of components of the flagellar T3S system. The membrane-spanning basal body consists of two OM rings (L and P rings) that are connected via a distal and proximal rod to the IM ring (MS ring). The MS ring is surrounded by 8 to 11 stator complexes in the IM that provide proton-conducting channels and is associated with the export apparatus, the C ring, the ATPase, and the regulator of the ATPase. The architecture of the export apparatus and of the cytoplasmic components of the flagellar T3S system is probably similar to that in translocation-associated T3S systems (see Fig. 2). Structures that are different from those in translocation-associated T3S systems are indicated.

OUTER APPEARANCE—EXTRACELLULAR APPENDAGES OF FLAGELLAR AND TRANSLOCATION-ASSOCIATED T3S SYSTEMS

Needles, Pili, and Flagella—Protein Transport Channels and Motility Organelles

Translocation-associated and flagellar T3S systems are associated with extracellular appendages that differ significantly in their structures and compositions. The basal body of translocation-associated T3S systems is linked to an extracellular pilus (plant pathogens) or a needle (animal pathogens) (Fig. 2) that contains an inner channel through which secreted proteins might be transported. The pilus from plant pathogens is up to 2 μm long and presumably spans the plant cell wall, which is a major obstacle for the transport of bacterial effector proteins into plant cells (59, 233, 248, 315, 463, 568, 594). The T3S needle from animal pathogens is significantly shorter than the pilus from plant pathogens and often has a determined length, which varies from 40 to approximately 80 nm in different pathogens (45, 224, 257, 291, 540). Needle assembly is probably initiated in the periplasm and occurs at the tip of the needle (444, 478). Since purified needle proteins (members of the YscF family) build structures of several micrometers, the regulation of needle length likely depends on the context of the T3S system (444, 447) (see below).

Nuclear magnetic resonance (NMR) and crystal structure analyses of needle proteins suggest that they form a hairpin-like structure with a central head region that connects the helical N- and C-terminal regions (131, 448, 530, 585, 630). While the central head region is presumably located at the needle surface, the C-terminal helix is buried in the needle wall and might undergo a conformational change upon polymerization (131, 444). Interestingly, needle components share structural similarities with components of the flagellar filament, although they do not share sequence similarities at the amino acid level. Thus, the needle subunit MxiH from *Shigella* spp. assembles into a helical structure with 5.6 subunits per turn, which is similar to the helical symmetry of the flagellar filament (approximately 5.5 subunits per turn) (103, 616). Furthermore, crystal structure analysis of the needle protein PrgI and the needle tip protein (see below) SipD from *Salmonella* spp. revealed that five molecules of PrgI assemble with five molecules of SipD to form the needle tip (337). However, the symmetry of the needle from *Salmonella* spp. is apparently highly

variable, with an average of approximately 6.2 subunits per turn (191). Structural rearrangements in the needle might therefore occur and could contribute to the transmission of signals such as host cell sensing from the tip of the needle to the base (see below). This hypothesis is supported by the finding that several mutations in needle proteins lead to constitutive T3S (102, 131, 268, 552, 569).

In addition to the needle, T3S systems from some gastrointestinal pathogens (enterohemorrhagic *E. coli*, EPEC, and *Citrobacter rodentium*) contain a filament structure on top of the needle that is composed of the filament protein EspA. The EspA filament might connect the needle to the translocon (see below) and encloses an inner channel with a diameter of 2 to 2.5 nm (117, 277, 497). Interestingly, EspA has a similar helical structure to that of the flagellar filament protein FliC (116, 277, 616). However, the EspA filament has a smaller external diameter (12 nm versus 24 nm for the flagellar filament), which could be caused by the smaller size of EspA (192 amino acids versus 494 amino acids for FliC) (116, 616). In addition to EspA filaments from gastrointestinal pathogens, a sheath-like surface appendage with a diameter of 30 to 70 nm and a highly variable length has also been observed for the SPI-2-encoded T3S system from *Salmonella enterica* serovar Typhimurium (83). The precise function and composition of this surface structure, however, have not yet been investigated.

In contrast to translocation-associated T3S systems, the flagellar T3S system is associated with an extracellular hook, which is composed of approximately 120 molecules of FlgE and has a length of 55 ± 6 nm (222). The hook is connected to the flagellar filament via the hook-filament junction proteins FlgK and FlgL, which are incorporated at the tip of the hook (228, 237) (Fig. 3). The filament consists of around 20,000 subunits of FliC and is 10 to 15 μm long. It terminates with a pentameric cap structure that is built by the filament cap protein FliD (616). *fliD* mutants are deficient in filament formation, suggesting that the filament cap is required for the assembly of FliC monomers to form a helical structure (227). EM studies revealed that the filament cap contains five leg-like anchor regions and provides a docking site for one FliC molecule. Rotation of the cap allows the entry of the next FliC molecule and thus could promote the folding and insertion of FliC monomers into the growing filament structure (615).

Port of Entry for Effector Proteins—the Translocon and the Tip Complex

The translocon. Translocation of effector proteins into the eukaryotic cell cytosol is mediated by a bacterial channel-like translocon, which is inserted into the host plasma membrane and usually consists of two hydrophobic proteins that are referred to as major (e.g., YopB, IpaB, SipB, and EspD, with two transmembrane helices) and minor (e.g., YopD, IpaC, SipC, and EspB, with one transmembrane helix) translocon proteins (Table 1). Translocon proteins act outside the bacterial cell; however, a *Yersinia yopB* null mutant cannot be *trans*-complemented upon coinfection with another *Yersinia* strain that delivers YopB (473). Similar observations were reported for the translocon proteins PopB and PopD from *Pseudomonas aeruginosa*, suggesting that translocon proteins act in *cis* (95). It is therefore assumed that the membrane insertion of translocon proteins is closely linked to the activity of the corresponding secretion apparatus.

Translocon proteins form a hetero-oligomeric protein complex of presumably six to eight subunits with an internal diameter of approximately 1.2 to 3.5 nm (44, 113, 236, 339, 387, 403, 490, 569). Recent crystal structure analysis of the translocon proteins IpaB from *S. flexneri* and SipB from *Salmonella* spp. revealed structural similarities to bacterial pore-forming toxins, which could indicate a common evolutionary origin of both protein families and similar mechanistics underlying membrane insertion (26). Experimental evidence reported for translocon proteins from animal-pathogenic bacteria suggests that the formation of a functional translocation channel depends on the composition of the host cell membrane. Thus, infection studies with *P. aeruginosa* revealed that some cell lines are resistant against T3S-mediated protein injection, indicating the requirement of certain host cell properties for efficient effector protein translocation (357, 471). This observation was supported by the finding that the alteration of the host plasma membrane composition renders cells insensitive against T3S by *P. aeruginosa* (55). It was already previously proposed that the formation of a functional T3S translocon occurs preferentially in specific microdomains of the host cell membrane that are rich in cholesterol and glycosphingolipids (490). These microdomains, also known as lipid rafts, are often involved in the attachment of invading bacterial pathogens, bacterial cytotoxicity, and contact-mediated T3S, as shown, for example, for *Bordetella* spp. and *S. flexneri* (158, 187, 563). Notably, depletion of cholesterol from host cell membranes impairs bacterial entry of *S. flexneri* (301) and affects effector protein translocation by *P. aeruginosa* and EPEC (13, 570). It is therefore assumed that cholesterol is required for the efficient formation of a functional translocation channel, while it is apparently dispensable for the membrane insertion of translocon proteins *per se*, as shown for *P. aeruginosa* (490, 570).

In agreement with the predicted contribution of cholesterol to channel formation is the finding that the translocon proteins PopB, SipB, and IpaB, from *P. aeruginosa*, *Salmonella* spp., and *S. flexneri*, respectively, can bind cholesterol (172, 216). Furthermore, IpaB from *S. flexneri* also associates with raft-containing liposomes (563) and binds to the transmembrane protein CD44, which preferentially localizes to lipid rafts and was previously shown to be recruited to the site of bacterial attachment during infection by EPEC (207, 301, 506). CD44 is a receptor for hyaluronic acid and other ligands and contains a cytoplasmic domain that interacts with actin cytoskeleton-associated proteins. In this context, it is interesting that the host actin cytoskeleton was proposed to contribute to translocon channel formation. Thus, the actin-depolymerizing agents cytochalasin D and latrunculin B were shown to inhibit the translocation of ExoS by the T3S system from *P. aeruginosa* (570). Furthermore, it was previously reported that deletion of the T3S effector gene *yopE* in *Yersinia pseudotuberculosis* resulted in increased amounts of translocated effector proteins and increased pore-forming activity that was dependent on actin polymerization (3, 572). The inhibition of pore formation by YopE was linked to its GTPase-activating activity, which modulates the activity of Rho GTPases and is required for the YopE-mediated depolymerization of actin filaments (361, 572, 576).

Inhibition of pore formation was also shown for the cysteine protease YopT, which cleaves Rho GTPases, as well as for the effector protein YopK (141, 225, 361, 500, 572, 640). Furthermore, an increase in the size of the translocation pore was observed for mutants of *Salmonella* spp. that were deprived of individual effector genes (95). This indicates that some effectors could have an

TABLE 1 Conserved components of flagellar and translocation-associated T3S systems[a]

Flagellar T3S system from *Salmonella* spp.	Translocation-associated T3S systems						Predicted functions/characteristics	Published structures	
	Animal-pathogenic bacteria				Plant-pathogenic bacteria				
	Yersinia spp.	*Salmonella* spp. (SPI-1)	EPEC	*S. flexneri*	*Xanthomonas* spp.	*P. syringae*		Protein(s)	Reference(s)
Cytoplasmic components									
FliI	YscN	InvC	EscN	Spa47	HrcN	HrcN	ATPase	EscN, FliI	239, 627
FliH	YscL	—	EscL (Orf5)	MxiN	HrcL	HrpE	Regulator of the ATPase		
FliM, FliN, FliG	YscQ	SpaO	EscQ (SepQ)	Spa33	HrcQ	$HrcQ_A$ + $HrcQ_B$	Predicted C ring component, possibly involved in substrate docking	$HrcQ_B$, FliM, FliG-FliM, FliG, FliN	60, 61, 170, 429, 436
IM components of the export apparatus									
FlhB	YscU	SpaS	EscU	Spa40	HrcU	HrcU	Autocatalytic cleavage at the NPTH motif; cytoplasmic domain is presumably involved in the substrate specificity switch and in substrate recognition	$EscU_C$, $SpaS_C$, $YscU_C$, $Spa40_C$	130, 334, 597, 626
FlhA	YscV	InvA	EscV	MxiA	HrcV	HrcV	Cytoplasmic domain is possibly involved in substrate docking	$FlhA_C$, $InvA_C$	23, 320, 388, 474, 606
FliP	YscR	InvL/SpaP	EscR	Spa24	HrcR	HrcR			
FliQ	YscS	SpaQ	EscS	Spa9	HrcS	HrcS			
FliR	YscT	InvN/SpaR	EscT	Spa29	HrcT	HrcT			
	YscD	PrgH	EscD	MxiG	HrcD	HrcD	Not highly conserved; forms multimeric ring structures	PrgH, MxiG, $YscD_N$	25, 335, 348, 356, 516
FliO	—	—	—	—	—	—			
Periplasmic/IM-associated components									
FliF (MS ring)	YscJ	PrgK	EscJ	MxiJ	HrcJ	HrcJ	Forms multimeric ring structures; lipoprotein	EscJ, PrgK	112, 348, 614
FlgB, FlgC, FlgF, FlgG	YscI	PrgJ	EscI (rOrf8)	MxiI	HrpB2	—	Predicted inner rod protein		
OM components									
—	YscC	MxiD	EscC	InvG	HrcC	HrcC	Secretin; forms a multimeric channel in the OM	YscC, EscC, InvG	67, 108, 516
—	YscW	MxiM	—	InvH	—	—	Pilotin	MxiM, MxiD-MxiM	305, 417
FlgI	—	—	—	—	—	—	P ring		
FlgH	—	—	—	—	—	—	L ring		
Extracellular components									
FlgJ	—	—		—	—	—	Rod cap protein	FlgJ	214, 270
FlgE	—	—		—	—	—	Hook protein		
FlgD	—	—		—	—	—	Hook cap		
FlgL, FlgK	—	—		—	—	—	Hook-filament junction proteins		
FliC	—	—		—	—	—	Filament protein	FliC	616
FliD	—	—		—	—	—	Filament cap	FliD	615
—	YscF	PrgI	EscF	MxiH	HrpE	HrpA	Pilus or needle proteins	YscF-YscE-YscG complex, PrgI, MxiH, PrgI-SipD, PscF-PscE-PscG complex, BsaL (*Burkholderia pseudomallei*)	103, 131, 337, 444, 448, 530, 585, 630

—	LcrV	SipD	—	IpaD	—	—	Needle tip proteins	LcrV, SipD, IpaD, BipD	87, 140, 252, 337, 424
—	YopB, YopD	SipB, SipC	EspD, EspB	IpaB, IpaC	HrpF, XopA	HrpK	Translocon proteins	EspB, EspD, IpaB, IpaB-IpgC, PcrH-PopD, SipB, PrgI-SipD	26, 163, 236, 249, 274, 337, 338
			EspA	—	—	—	Filament on top of the needle	EspA	613
—	—	—	—	—	—	—			

[a] Proteins from flagellar and translocation-associated T3S systems with similar functions and/or homologous sequences are given in the same row. Alternative protein names are given in parentheses. —, no homologous or functionally equivalent proteins have been identified.

anti-pore-formation activity after being translocated into the host cell. The predicted inhibitory activity on pore formation could impose a feedback regulation on effector protein translocation and might ensure that all infected cells contain similar levels of effector proteins and are not killed too rapidly by an effector overdose (562). Furthermore, the negative control of pore formation by translocated effector proteins might also counteract the proinflammatory responses of the host cell that are activated in the presence of the translocation pore. Previous mutant studies of *Yersinia* spp. revealed that it is the presence of the T3S translocon that triggers proinflammatory responses, which are in turn suppressed by translocated effector proteins (503). This predicted feedback regulation might itself be controlled by the degradation of effector proteins inside the host cell, as shown for YopE from *Yersinia* spp., which is degraded by the eukaryotic ubiquitination machinery. Because the accumulation of a degradation-resistant YopE mutant derivative leads to reduced translocation of effector proteins into the host cell, it was proposed that the pathogen exploits the host proteasome to indirectly regulate effector protein delivery (194, 470). Ubiquitination and proteasome-mediated degradation were also reported for the effector proteins SopE and SopB from *Salmonella* spp. (276, 290).

Compared to the case for animal-pathogenic bacteria, translocon proteins from most plant-pathogenic bacteria have been studied less intensively, and the precise composition of the translocation channel still remains to be investigated. In *Xanthomonas* spp., effector protein translocation depends on HrpF, which is a predicted component of a channel-like protein complex (74). Interestingly, translocation of individual effector proteins appears to be reduced upon recognition of the effector protein AvrBs2 by the corresponding pepper resistance protein Bs2, which initiates plant defense responses (631). The molecular mechanisms underlying this apparent feedback control are unknown. In contrast to *Xanthomonas campestris* pv. vesicatoria, several predicted translocon proteins from other plant-pathogenic bacteria are not essential for pathogenicity, suggesting that additional proteins such as harpins are involved in effector protein translocation (47, 300, 364, 438). Harpins are small, heat-stable T3S substrates from plant-pathogenic bacteria that are rich in glycine and can elicit plant defense responses when infiltrating the plant apoplast at high concentrations. In line with the predicted role of harpins in effector protein translocation is the finding that the harpin protein HrpZ from *P. syringae* forms transmembrane channels and assembles into oligomeric structures that consist of at least 16 molecules of HrpZ (155, 211, 307, 308). Alternatively, however, some harpin proteins can also target the plant cell wall (84, 174, 317).

The tip complex. The T3S translocon from animal-pathogenic bacteria is presumably connected to the needle by a tip complex that was initially visualized by scanning transmission EM studies of needles from *Yersinia enterocolitica* (396). The tip complex might serve different purposes, including sensing of the host cell contact, control of T3S, and insertion of the translocon into the host plasma membrane.

The tip complex from *Yersinia* spp. consists of five molecules of the hydrophilic LcrV protein that oligomerize *in vitro* and form ring-like structures with an internal diameter of 3 to 4 nm (63, 197, 393, 395, 396). The hydrophobic translocon proteins YopB and YopD from *Yersinia* spp. probably do not participate in tip

complex formation. This is in contrast to the tip complex from *S. flexneri*, which contains one molecule of the translocon protein IpaB, which forms a complex with four hydrophilic molecules of the tip protein IpaD (46, 164, 252, 418, 569). It was proposed that IpaB and IpaD plug the needle prior to host cell contact and are thus involved in the regulation of T3S (362). In agreement with this model, deletion of *ipaB* and *ipaD* leads to constitutive T3S *in vitro* (432, 442, 461, 501, 569). Similar findings were reported for a *P. aeruginosa* mutant lacking the predicted tip complex protein PcrV (310, 407). However, the deregulation of T3S in the absence of a tip protein does not appear to be a general phenomenon, because it was not observed for a *Yersinia* sp. *lcrV* mutant (32, 481). It is assumed that a conformational change in the tip complex upon completion of the translocon is transduced via the needle subunits to the base of the T3S system and activates the secretion of effector proteins (131, 569). Alternatively, recent experimental evidence reported for *Salmonella* spp. suggests that the secretion of effector proteins could also be activated upon a shift in the extracellular pH that is sensed by the needle (623) (see below).

ARCHITECTURE OF THE BASAL BODY AND EXPORT APPARATUS IN TRANSLOCATION-ASSOCIATED AND FLAGELLAR T3S SYSTEMS

Translocation-Associated and Flagellar T3S Systems Contain Different OM Ring Components

The OM rings of translocation-associated T3S systems are built by proteins belonging to the secretin family (Table 1), whose members also participate in the assembly of type II secretion systems and type IV pili but are absent from flagellar T3S systems. Secretins consist of an N-terminal domain with a cleavable signal sequence that directs the protein for Sec-dependent transport across the IM into the periplasm. The N-terminal region of T3S secretins is not highly conserved among different species and might form a periplasmic neck structure that connects the secretin channel to components of the IM ring (223, 492). The C-terminal membrane-spanning region of secretins multimerizes to form OM rings with a diameter of approximately 11 nm and a 12- to 14-fold symmetry (66, 108, 223, 286, 516). A 15-fold symmetry was recently reported for the OM ring of the translocation-associated T3S system from *S.* Typhimurium (493). Oligomerization and channel formation by secretins are often mediated by pilotins, which are small OM lipoproteins with limited sequence homology that have been identified in animal-pathogenic bacteria (e.g., see references 66, 108, 114, and 495). In the absence of their cognate pilotins, secretins localize to the IM, as shown for InvG from *Salmonella* spp. (114) and YscC from *Yersinia* spp. (66). Interestingly, experimental evidence for the presence of pilotins in plant-pathogenic bacteria is missing.

In contrast to the secretins of translocation-associated T3S systems, flagellar T3S systems contain an L (lipopolysaccharide) ring in the OM, consisting of the lipoprotein FlgH (254, 491) (Fig. 3 and Table 1). The L ring is associated with a periplasmic P (peptidoglycan) ring, which is composed of 26 copies of FlgI (229, 254, 255). L and P rings form a stiff structure that serves as bushing for the rotating rod of the flagellar T3S system and is absent from flagellar T3S systems of Gram-positive bacteria that do not possess an OM.

Is the Predicted Periplasmic Rod Structure a Building Platform for the Needle or the Pilus?

EM studies of isolated needle complexes from *S.* Typhimurium revealed the presence of an internal channel of the basal body, localized in the periplasm, which was referred to as the inner rod and is composed of PrgJ (347, 348). The inner rod of the T3S system from *Salmonella* spp. is connected by a socket-like structure to the IM rings and might be required for stable anchoring of the extracellular needle, which probably protrudes into the periplasm as revealed by single-particle EM (347, 348, 478, 605). Experimental evidence for an inner rod structure was also reported for the T3S system from EPEC as well as for flagellar T3S systems (410), but the presence of this structure has not yet been confirmed.

It is assumed that the predicted inner rod of translocation-associated T3S systems is composed of multiple copies of a single subunit (e.g., YscI from *Yersinia* spp.; note that predicted inner rod proteins are not highly conserved). In contrast, the inner rod of flagellar T3S systems consists of four different components that build up the proximal rod (composed of FlgB, FlgC, and FlgF) and the distal rod (composed of FlgG) (230). The latter is surrounded by the P and L rings. The assembly of the predicted rod depends on FlgJ, a protein with a dual function. The N-terminal domain of FlgJ serves as a rod-capping protein that probably assists in the formation of the inner rod, while the C-terminal domain of FlgJ acts as a muramidase. The muramidase activity of FlgJ might be involved in the degradation of peptidoglycan and thus could be required for the efficient assembly of the rod structure in the periplasm (220, 401). Since the diameter of the flagellar T3S system-associated ring structures has been estimated to be approximately 11 nm (514) or 7.5 nm (534), the system is too large to pass through the natural pores of peptidoglycan, which are approximately 2 nm wide (135). The assistance of peptidoglycan-degrading enzymes is therefore often required to facilitate the assembly of membrane-spanning high-molecular-weight protein complexes such as flagellar or translocation-associated T3S systems (also see below).

The IM Ring Components of Translocation-Associated and Flagellar T3S Systems Differ in Their Complexity

It is assumed that the predicted inner rod is associated with IM rings of the T3S system that differ in complexity. While the IM rings (or MS rings [membrane and supramembranous rings]) of flagellar T3S systems are composed of FliF (Table 1) (34, 535, 560), the IM rings of translocation-associated T3S systems consist of at least two proteins, including members of the YscJ family of lipoproteins and the less conserved YscD family (Table 1). YscJ family members form a ring structure that is located at the periplasmic site of the IM and is presumably attached to the membrane by the N-terminal lipid moiety of YscJ and homologs. Several YscJ family members also contain a predicted C-terminal transmembrane helix (10, 492, 504). YscD and homologs are lipoproteins with an N-terminal cytoplasmic and a C-terminal periplasmic domain and might form a multimeric ring structure next to the YscJ ring (25, 142, 293, 492, 516). Protein-protein interaction studies revealed that YscJ and YscD family members interact not only with each other but also with members of the YscC family of OM secretins (142, 410, 467, 479) (Table 2). Interestingly, the YscJ and YscD homologs EscJ and EscD from EPEC were also shown to

TABLE 2 Interaction partners of T3S system components

Type of component and protein family	Homolog(s) (organism)[a]	Interacting T3S system component(s)	Interacting T3S substrate(s) and/or chaperone(s)	Reference(s)
Predicted C ring components				
YscQ family	YscQ (*Yersinia* spp.)	YscL, YscK, YscN, $YscU_C$, $YscQ_C$	YscP (T3S4 protein)	77, 245, 457, 458
	SpaO (*Salmonella* spp., SPI-1)	OrgA, OrgB (probably required for efficient assembly and/or stability of SpaO complex), InvC	Translocon (SipB, SipC, SipD) and effector proteins (SipA, SptP) associate with SpaO-OrgA-OrgB complex; association depends on the YopN homolog InvE and the chaperone SicA (for translocon proteins) as well as on the CBD of SptP	304, 515
	SsaQ (*Salmonella* spp., SPI-2)	$SsaQ_S$ (generated by tandem translation of $ssaQ_L$)		622
	Spa33 (*S. flexneri*)	Spa47, MxiN, MxiK, MxiG, MxiJ	Effector proteins VirA, IcsB, IpaC, and IpgB1; T3S4 protein Spa32	251, 256, 390
	EscQ (EPEC)	EscN, EscL		33
	CdsQ (*Chlamydia trachomatis*)	CdsD, CdsS, CdsT	Mcsc (chaperone of effector Cap1), complex of Cap1 and Mcsc	304
	CdsQ (*Chlamydia pneumoniae*)	CdsQ, CdsD, CdsL, CdsN, FlhA*		250, 515, 524, 525
	$HrcQ_A$ (*P. syringae*)	$HrcQ_B$		170, 550
	$HrcQ_B$ (*P. syringae*)	$HrcQ_A$		170, 550
	FliM*		FliJ (chaperone-binding protein)*	206
	FliN*	FliH*		206, 358
	FliG*	FliF*		419
ATPases				
YscN family	YscN (*Yersinia* spp.)	YscL, YscK, YscQ	YscP (T3S4 protein that interacts with YscN-YscL-YscQ complex), YscF (needle protein), YscE and YscG (chaperones of YscF), YopR (effector protein)	42, 123, 245, 458, 459, 510
	InvC (*Salmonella* SPI-1)		SicP (chaperone of SptP), SopD (effector)	4, 49
	SsaN (*Salmonella* SPI-2)		SrcA (chaperone of SseL and PipB2), SsaE (chaperone of SseB)	101, 369
	Spa47 (*S. flexneri*)	Spa33, MxiK, MxiN	MxiC (secreted regulator, YopN homolog; the N terminus of MxiC is required for the interaction)	51, 251, 256
	EscN (EPEC)	EscL, EscQ	Tir (effector), CesT (class IB chaperone)	33, 195, 546
	CdsN (*C. pneumoniae*)	CdsL, CdsD, CdsQ	CopN (YopN homolog and effector protein)	250, 515, 525
	HrcN (*X. campestris* pv. vesicatoria)	HrcN, HrcL, $HrcU_C$, HpaC (T3S4 protein)	HpaB (class IB chaperone)	329
	HrcN (*P. syringae*)	HrcN, HrpE (YscL family member)		550
	FliI* (*C. pneumoniae*)	CopN, CdsL, $FlhA_C$*		524
	FliI* (*Salmonella* spp.)	FlhA*, FlhB*, FliI*, FliH*	FliE*, FlgB*, FlgE*, FlgD* (rod/hook proteins), FlgK*, FlgL*, FliC* (filament proteins), FliJ* (chaperone-binding protein), FlgN* (chaperone; binds to FliI in complex with the substrate FlgK), FliT* (chaperone of filament-capping protein FliD)	205, 238, 382, 383, 544, 638
Putative negative regulators of ATPases				
YscL family	YscL (*Yersinia* spp.)	YscN, YscQ, $YscU_C$	YscP (T3S4 protein; interacts with YscN-YscL-YscQ complex)	42, 245, 457, 458
	EscL (EPEC)	EscN, EscQ	EspA (filament protein)	33, 288
	CdsL (*C. pneumoniae*)	CdsD, CdsN, CdsQ, FliI*, FlhA*		250, 515, 524, 525
	HrcL (*X. campestris* pv. vesicatoria)	HrcN, HrcU		329
	HrpE (*P. syringae*)	HrcN		550
	FliH* (*Salmonella* spp.)	FlhA*, FlhB*, FliI*, FliN*	FlgB*, FlgE*, FlgD* (rod/hook proteins), FlgK*, FlgL*, FliC* (filament proteins), FliJ* (chaperone escort protein)	185, 205, 358, 382, 383, 638
Components of the export apparatus				
YscU family	$YscU_C$ (*Yersinia* spp.)	YscL, YscK, YscQ		457
	Spa40 (*S. flexneri*)		Spa32 (T3S4 protein)	50
	EscU (EPEC)	EspR	EspD (translocon protein), EscI (predicted inner rod protein)	110, 475
	CdsU (*C. pneumoniae*)	FlhA*		524
	HrcU (*X. campestris* pv. vesicatoria)	HrcL	HpaB (class IB chaperone)	329
	$HrcU_C$ (*X. campestris* pv. vesicatoria)	HrcN, HpaC (T3S4 protein)	HrpB2 (early T3S substrate)	329, 330, 332
	HrcU (*P. syringae*)		HopAH1	550

(Continued on following page)

TABLE 2 (Continued)

Type of component and protein family	Homolog(s) (organism)[a]	Interacting T3S system component(s)	Interacting T3S substrate(s) and/or chaperone(s)	Reference(s)
	$FlhB_C$* (*Salmonella* spp.)	FliI*, FliH*, $FlhA_C$*	FliE*, FlgB*, FlgE*, FlgD* (rod/hook proteins), FlgK* (filament-type protein, weak interaction), FliK* (T3S4 protein)	381, 383, 386, 392
	$FlhB_{CN}$* (*Salmonella* spp.)	$FlhB_{CC}$*		381
YscV family	YscV (*Yersinia* spp.)	YscC (the interaction with YscC is reduced in the absence of YscD or YscJ), YscD (YscC is required for the interaction with YscD), YscJ (YscR, YscS, and YscT are required for the interaction with YscJ)		143
	$FlhA_C$* (*Bacillus subtilis*)		FliC (flagellin), FliD (filament cap; binds in complex with chaperone FliT)*, FliJ (chaperone-binding protein)*	23
	FlhA* (*C. pneumoniae*)	FliF*, CdsU, CdsL		524
	FlhA* (*Salmonella* spp.), $FlhA_C$* (*Salmonella* spp.)	FliH*, FliI*, FliF*, $FlhB_C$*, $FlhA_C$*	FlgE*, FlgD* (rod/hook proteins), FlgK*, FlgL* (hook-filament junction proteins; interaction with $FlhA_C$ is enhanced by the chaperone FlgN*), FliC* (filament protein), FliJ* (chaperone-binding protein), FlgN* (chaperone)	185, 359, 379, 383, 638
	FlhA* (*C. pneumoniae*)	CdsL, CdsU, CdsQ	SepZ (effector)	515, 524
YscR family	EscR (EPEC)	EscR, EscS, EscU	EspD (translocon protein)	110
YscS family	EscS (EPEC)	EscR, EscS	EspD (translocon protein)	110
IM ring components				
YscJ family	YscJ (*Yersinia* spp.)	YscJ, YscC, YscD		142, 467
	EscJ (EPEC)	EscC	EscF (needle protein)	410
	PrgK (*S. enterica*)	PrgH		479
YscD family	YscD (*Yersinia* spp.)	YscD, YscJ, YscC		142, 467
	PrgH (*S. enterica*)	PrgK		479
	CdsD (*C. pneumoniae*)	CdsQ, CdsL, CdsN		250, 525
	EscD (EPEC)	EscC	EscF (needle protein)	110, 410
	MxiG (*S. flexneri*)	Spa33 (MxiG interacts with phosphorylated peptide of Spa33)		25
MS ring components	FliF*	FlhA*, FliG		419, 524
OM ring components				
YscC family	YscC (*Yersinia* spp.)	YscJ, YscD		142, 467
	EscC (EPEC)	EscD	EscF (needle protein), EscI (predicted inner rod protein)	110, 410, 475
Needle/pilus components				
YscF family	YscF (*Yersinia* spp.)	YscN		123
	EscF (EPEC)	EscC, EscJ, EscD	EscF (needle protein)	410
	MxiH (*S. flexneri*)		IpaD (translocon protein)	629
	PrgI (*Salmonella* spp.)		SipD (translocon protein)	451

[a] Proteins from flagellar T3S systems are marked with asterisks.

interact with the needle protein EscF, suggesting that the IM rings might provide a connection not only to the secretin channel but also to the needle, which possibly sits atop the predicted periplasmic inner rod structure and thus protrudes into the periplasm (410) (see above and Table 2). In agreement with this hypothesis, needle-like structures were observed in isolated T3S needle complexes from *S.* Typhimurium that lacked the OM secretin (492).

Cryo-EM studies revealed that the symmetry of the IM rings of isolated translocation-associated T3S systems from *S. enterica* and *S. flexneri* ranges from 19- to 22- or 24-fold (19, 347, 478, 493). Alternatively, a 12-fold symmetry was proposed for IM rings from *S. flexneri* (223), whereas a 24- to 26-fold symmetry was observed for the IM rings of flagellar T3S systems (535, 542). The model of a 24-subunit ring model for IM rings of translocation-associated T3S systems was supported by the results of crystal structure analyses of the YscJ homolog EscJ from EPEC and the YscD homolog MxiG from *S. flexneri* (356, 614).

Although the constituents of IM and OM rings, including members of the YscC, YscJ, and YscD families, do not share significant amino acid similarities, crystal structure analyses of EscC, EscJ, and PrgH (Table 1) revealed a common $\alpha_2\beta_3$ fold that was also identified in the OM secretins GspD and DotD, from type II and type IV secretion systems, respectively, and was proposed to act as a ring-building motif (284, 400, 516, 614). However, deletion of the predicted ring-building motif in the PrgH homolog YscD did not affect the activity of the T3S system (467). The $\alpha_2\beta_3$ motif was also found in the C-terminal domain of the YscV homolog InvA, which is a component of the export apparatus (320). It is therefore conceivable that ring formation is a common characteristic of IM- and OM-associated components of the T3S sys-

tem. Notably, YscV and its flagellar homolog FlhA were reported to oligomerize, and it was assumed that approximately 20 subunits of FlhA are incorporated into the flagellar export apparatus, where they might form a ring structure outside the MS ring (143, 316). In future studies, it remains to be investigated whether the assembly of the export apparatus in the IM does indeed involve the formation of ring structures.

Transmembrane Components of the Export Apparatus Are Involved in Substrate Recognition

The IM rings of the needle complex most likely provide a scaffold for the assembly of the transmembrane components of the export apparatus that enclose the transport channel for secreted proteins. The export apparatus of translocation-associated T3S systems is composed of members of the YscR, YscS, YscT, YscU and YscV families, which presumably form a multimeric protein complex. In flagellar T3S systems, these proteins include members of the FlhA, FlhB, FliO, FliP, FliQ, and FliR protein families (summarized in Table 1). Components of the export apparatus contain one to eight transmembrane helices and differ in size and in the presence of cytoplasmic domains. Members of the YscU/FlhB and YscV/FlhA families of IM proteins contain two large cytoplasmic domains that were proposed to be involved in the recognition of secreted proteins (11, 23, 31, 379, 381, 383). In agreement with this hypothesis, the C-terminal domains of FlhB and FlhA from flagellar T3S systems were shown to interact with extracellular components of the flagellum (Table 2). Furthermore, an interaction was reported between the C-terminal domain of the YscU/FlhB homolog HrcU, from the plant-pathogenic bacteria *Xanthomonas campestris* pv. vesicatoria and *P. syringae*, and secreted proteins (332, 550). The contribution of YscU/FlhB family members to the substrate specificity switch is discussed below. It should be noted that in addition to the cytoplasmic domains of members of the export apparatus, the presence of substrate docking sites was also described for the ATPase and the predicted C ring (Table 2; see below). It therefore cannot be excluded that T3S systems contain multiple substrate docking sites and that different acceptor sites recognize different types of T3S substrates.

Power Supplies—the Cytoplasmic ATPase and the Flagellar Motor

The ATPase of the T3S system—key player or useful substituent? The export apparatus of flagellar and translocation-associated T3S systems is associated with a cytoplasmic ATPase which is a member of the YscN protein family (YscN/InvC/Spa47/EscN) and forms homo- or double-hexameric ring structures with an internal diameter of approximately 2.5 to 3 nm (96, 235, 267, 397, 445). Oligomerization and membrane contact of YscN family members lead to an increase of the ATPase activity, which is predicted to provide the energy needed for the secretion process (18, 21, 96, 378, 445, 627). Oligomerization of ATPases can also be induced upon binding of a T3S chaperone, as shown for the multicargo T3S chaperone SrcA from *Salmonella* spp., which interacts with the SPI-2-encoded ATPase SsaN (101) (Table 2). T3S chaperones are cytoplasmic proteins that bind to one or several T3S substrates and promote their stability and/or secretion (see below). Since ATPases of flagellar and translocation-associated T3S systems interact with effectors and/or effector-chaperone complexes, they were proposed to be involved in T3S substrate recognition (4, 195, 329, 510, 544, 546). Experimental evidence suggests that the ATPase dissociates T3S substrates from their cognate chaperones (4) and contributes to the unfolding of secreted proteins prior to their entry into the secretion apparatus (4). This is probably important for efficient secretion, because the inner channel of the T3S system has a diameter of 2 to 3 nm, which is too narrow to allow the passage of fully folded proteins (4, 45, 600).

Interestingly, experimental evidence reported for *Yersinia* spp. suggests that T3S can also occur in the absence of a functional ATPase, albeit in reduced amounts, and might then be driven by the proton motive force (PMF) (599). The PMF refers to the electrochemical potential difference of protons across a membrane and consists of the electrical potential difference ($\Delta\Psi$) and the proton concentration difference (ΔpH). PMF was also shown to contribute to flagellar T3S in *Salmonella* spp. in the absence of the ATPase FliI and its regulator FliH (376, 384, 435). It was therefore proposed that the PMF drives protein transport across both membranes, whereas the ATPase is required for the efficient initial docking of T3S substrates to the secretion channel. Notably, however, evidence for ATPase-independent secretion could not be observed for the plant-pathogenic bacterium *X. campestris* pv. vesicatoria (329), suggesting that the contributions of different energy sources to T3S can vary among plant- and animal-pathogenic bacteria.

Crystal structure analyses of the T3S-associated ATPase EscN from EPEC and the flagellar T3S-associated ATPase FliI revealed a structural similarity with the α/β subunits of F_oF_1-ATPases (239, 627). F_oF_1-ATPases consist of a membrane-spanning F_o domain and a solvent-exposed F_1 domain that rotate in opposite directions (Fig. 4). The F_1 domain is composed of a hexamer of α and β subunits arranged around a central stalk. A second peripheral stalk, which contains b and δ subunits, connects the F_o and F_1 domains. Interestingly, components of the second stalk share sequence homology with members of the YscL protein family that interact with the ATPase and are predicted regulators of its enzymatic activity (425) (Table 2; Fig. 4). A negative regulator might prevent ATP hydrolysis prior to the activation of the secretion system (382). FliH forms a $FliH_2$-FliI complex with the ATPase and presumably promotes the docking of FliI to the secretion apparatus at the IM (21, 205, 382). The absence of FliH leads to a loss of bacterial motility, but the phenotype of *fliH* mutants can be suppressed upon overexpression of FliI or upon introduction of point mutations into the cytoplasmic domain of FlhA or FlhB (373). Since FlhA and FlhB interact with FliI and FliH (Table 2), mutations in the cytoplasmic domains of both proteins probably promote the docking of the ATPase complex to the export apparatus in the absence of FliH (358, 359, 383, 638). These findings suggest that FliH *per se* is not essential for flagellar T3S. Taken together, the data indicate that the docking of the ATPase and its enzymatic activity are important for T3S but are probably not the only energy source of the T3S system.

The flagellar ATPase complex interacts with the chaperone-binding protein FliJ. FliH, the regulator of the ATPase of flagellar T3S systems, interacts not only with the ATPase FliI but also with the soluble FliJ protein, which is an essential cytoplasmic component of the secretion machinery and contributes to the secretion of rod, hook, and filament proteins (185, 205, 372). Interestingly, analysis of the crystal structure of FliJ from *S. enterica* revealed a structural similarity with the γ subunit of the F_oF_1-ATPase, which is part of the central stalk of the soluble F_1 domain (235) (see above and Fig. 4). Furthermore, the results of cryo-EM and protein-

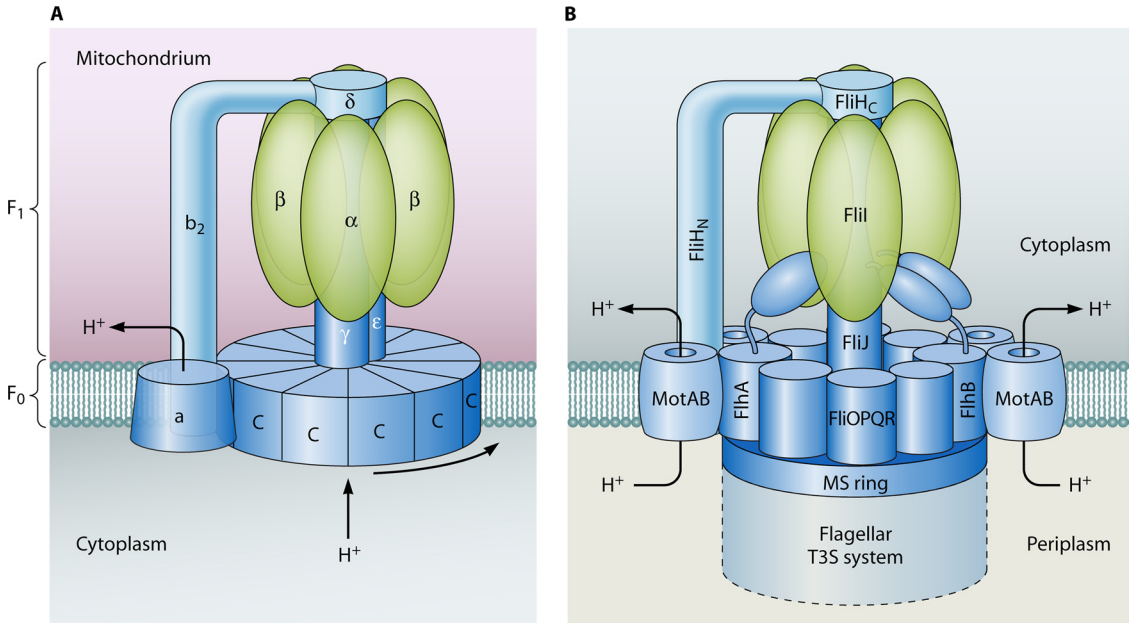


FIG 4 Similarities between F_oF_1-ATPases and T3S-associated ATPases. (A) Model of the F_oF_1-ATPase. The F_oF_1-ATPase consists of a membrane-embedded F_o domain and a catalytic F_1 domain. The F_1 domain is composed of an $\alpha_3\beta_3$ hexamer and is associated via the central stalk (consisting of the γ and ε subunits) and the peripheral stalk (composed of δ, b_2, and a subunits) with the F_o domain. The F_o domain contains a and b (not shown) subunits and 12 c subunits that form proton-conducting channels. The energy provided by the proton influx drives the rotation of the F_o domain and ATP synthesis. (B) Model of the flagellar T3S-associated ATPase FliI and its interaction partners. FliI presumably forms a hexameric complex that is associated with the regulator of the ATPase, FliH, which shares structural similarity with the peripheral stalk of the F_oF_1-ATPase. Structural similarity was also reported for the chaperone-binding protein FliJ and components of the central stalk of the F_oF_1-ATPase (235). FliI associates with the export apparatus of the T3S system, which is connected to the MS ring in the IM and is surrounded by stator complexes (also see Fig. 2 and 3). Note that the organization of the ATPase complex is speculative and that the central position of FliJ in the ATPase ring has not been confirmed experimentally. The cytoplasmic C ring is not shown in this model.

protein interaction studies suggest that FliJ inserts into the central channel of the ATPase ring and promotes the formation of a hexameric ATPase ring when mixed with FliI at a ratio of 6:1 (FliI:FliJ) (235) (Table 2). Given the previous finding that ring formation by FliI increases the ATPase activity (96, 378), FliJ could be involved in the activation of ATPase-driven T3S. However, as mentioned above, flagellar T3S can also occur in the absence of the ATPase and is then driven by the PMF, which consists of $\Delta\Psi$ and ΔpH. A recent publication revealed a contribution of FliJ to the $\Delta\Psi$-driven export of flagellar T3S substrates that probably depends on the interaction of FliJ with the linker region of the cytoplasmic domain of the IM protein FlhA ($FlhA_C$) (384). In agreement with this hypothesis, the binding sites of FliJ in FlhA were shown to be required for the functioning of both proteins, and *vice versa*. The authors therefore proposed that FliJ alters the conformation of $FlhA_C$ to activate $\Delta\Psi$-driven protein export (384).

Notably, however, the role of FliJ appears to be more complex. An alternative function of FliJ as a chaperone-binding protein was described because FliJ also interacts with the chaperones FlgN (chaperone of the hook-filament junction proteins FlgK and FlgL) and FliT (chaperone of the filament cap protein FliD) (23, 166). Similarly, the FliJ orthologs InvI and YscO, from *Shigella* spp. and *Yersinia* spp., respectively, interact with T3S chaperones (165). In the case of FliJ, experimental evidence suggests that FliJ promotes the interaction of the FliT-FliD complex with $FlhA_C$ (23). The interaction of the hook-filament junction protein FlgK with $FlhA_C$, however, appears to depend on its cognate chaperone, FlgN, rather than on the presence of FliJ (379). It therefore remains to be clarified whether FliJ and homologs play a general role in the docking of chaperone-substrate complexes to components of the export apparatus or if they are proteins with multiple functions that are also involved in the activation of ATPase- and/or PMF-driven T3S.

Flagellar rotation depends on membrane-embedded stator complexes. In addition to the ATPase, the activity of flagellar T3S systems depends on 8 to 11 membrane-embedded stator complexes, which consist of MotA and MotB and are absent from translocation-associated T3S systems. MotA contains four transmembrane helices and a cytoplasmic domain and interacts with the single-pass IM protein MotB, which is anchored by a peptidoglycan-binding domain in the bacterial cell wall (94, 128, 136). MotA and MotB form a hetero-oligomeric $MotA_4MotB_2$ complex that provides a channel for proton influx into the bacterial cytoplasm (41, 53, 483, 523) and converts the energy of the proton flux into a mechanical force that drives flagellum rotation (40, 279, 637) (Fig. 3). It has been estimated that a flow of approximately 1,200 protons is required for each rotation of the flagellar filament (360).

MotA interacts with the cytoplasmic FliG protein, which forms a ring of 26-fold symmetry on the cytoplasmic side of the MS ring and is directly involved in torque generation (183, 309, 325, 326, 535, 637). FliG is part of the switch complex that is required for flagellar rotation and the switching between clockwise and counterclockwise rotation. The switch complex (which corresponds to the predicted C ring [see below]) also consists of FliM and FliN, which form a pentameric FliM-$FliN_4$ complex (61, 179, 242, 434, 609, 632, 633). FliM is presumably located between FliG and FliN and contains a binding site for the signaling molecule phospho-

CheY, which promotes clockwise rotation of the flagellum (62, 375, 429, 434, 508, 595). A binding site for phospho-CheY was also identified in FliN, but FliN has a relatively minor role in flagellar switching and rotation (480). It is assumed that the proton flux across the $MotA_4MotB_2$ complexes induces a conformational change in the cytoplasmic domain of MotA and applies a force on the switch complex, most likely via electrostatic interactions between MotA and FliG (39, 279, 637). These electrostatic forces cause the rotation of the flagellar rotor, which consists of the predicted C ring, the IM rings, the periplasmic rod, the hook, and the flagellar filament and might rotate as one unit.

The Predicted Cytoplasmic C Ring of the T3S System Is a Potential Substrate Docking Site

FliM and FliN of flagellar T3S systems not only are involved in flagellar rotation but also form most of the cytoplasmic C ring that is associated with the IM ring complexes of the T3S system and contributes to the secretion process. The C ring is a cup-like structure with a diameter of approximately 40 nm that has been visualized by EM of isolated flagellar hook-basal body complexes and has a symmetry varying between 31- and 38-fold (179, 269, 542, 543, 619). The C ring of flagellar T3S systems is estimated to be composed of 34 copies of FliM and approximately 100 copies of FliN (436, 543, 633). The third component, FliG, interacts not only with the pentameric FliM-$FliN_4$ complex and MotA but also with the MS ring component FliF and could therefore couple the C ring to the transmembrane components of the flagellar T3S system (61, 179, 419, 633) (Table 2). Interactions have also been observed between FliN and the ATPase regulator FliH, as well as between FliM and the ATPase-associated chaperone-binding protein FliJ (see above), suggesting that the C ring is involved in the docking of the ATPase complex (206, 358) (Table 2). Notably, the phenotype of C ring mutants can be suppressed by enhanced levels of the ATPase FliI or the master regulator FlhDC. It was therefore concluded that the C ring *per se* is not essential for flagellum formation (160, 280).

Predicted C ring components of translocation-associated T3S systems include members of the YscQ protein family, which share amino acid sequence similarities with FliM and FliN. YscQ and homologs interact with effector proteins or effector-chaperone complexes and were therefore proposed to act as a recruitment platform for secreted proteins (390, 515) (Table 2). The purification of recombinant YscQ revealed that it exists as a complex of two proteins, including full-length YscQ and a shorter protein corresponding to the C-terminal portion of YscQ, designated YscQ-C and synthesized from an internal translation initiation codon in YscQ (77). A similar tandem translation was recently reported for the YscQ homolog SsaQ from the *Salmonella* sp. SPI-2 (622). Crystal structure analysis revealed that YscQ-C forms a homodimer and shares structural similarities with the C ring component FliN and the FliN homolog $HrcQ_B$ from the plant-pathogenic bacterium *P. syringae* (77). The C-terminal domain of $HrcQ_B$ is itself structurally similar to FliN and was shown to interact with $HrcQ_A$, which shares similarities with FliM (61, 170). In agreement with a predicted function as a cytoplasmic component of the T3S system, the YscQ homolog Spa33 from *S. flexneri* localizes to the cytoplasmic side of the T3S system and interacts with the IM ring components MxiG and MxiJ as well as with the ATPase Spa47 (390). Furthermore, YscQ and the homologous CdsQ protein from *Chlamydia* spp. interact with the ATPase of the T3S system and its predicted regulators, i.e., YscL and CdsL, respectively (245, 250, 515) (Table 2). It was therefore postulated that the C ring is also present in translocation-associated T3S systems. Notably, however, in contrast to those of flagellar T3S systems, predicted C rings of translocation-associated T3S systems have not yet been visualized by EM studies (223, 347, 348, 493). The existence of these specialized cytoplasmic ring structures in translocation-associated T3S systems therefore remains to be proven.

THE CONSTRUCTION PHASE—HOW THE BASAL BODY AND EXPORT APPARATUS ARE ASSEMBLED

Stepwise Assembly of the Membrane-Spanning Basal Body

Experimental evidence suggests that there is a hierarchy in the assembly of the membrane-spanning basal body. An analysis of the translocation-associated T3S system from *S.* Typhimurium suggested that the ring structures in the IM and OM are assembled prior to the inner rod (529). Since IM rings and needle-like structures were observed in the absence of the OM secretin (492), the OM ring is probably dispensable for the assembly of the IM structures and the needle. In agreement with this finding, overexpression of the IM ring components PrgH and PrgK in *E. coli* led to the formation of ring structures even in the absence of other components of the basal body (273). Mutant studies with *Salmonella* spp. revealed that the IM and OM ring structures of the needle complex are dispensable for the formation of the export apparatus in the IM. Thus, it was shown that SpaP, SpaQ, and SpaR (YscR, YscS, and YscT family members) can assemble into a stable complex even in the absence of the needle complex (577). It is therefore likely that the assembly of the export apparatus precedes needle complex formation in *Salmonella* spp.

An inside-outside assembly was proposed not only for the translocation-associated T3S system but also the flagellar T3S system from *Salmonella* spp. The assembly of the flagellar basal body presumably initiates with the insertion of the MS ring into the IM and is followed by the attachment of the C ring and the stator complexes. After C ring formation, the export apparatus, the periplasmic rod, and the P and L rings are built (292). A recent study suggested, however, that the formation of the MS ring is preceded by oligomerization of the IM component FlhA, which is part of the export apparatus and might thus be the first component of the flagellar T3S system that is inserted into the IM (316).

In line with the predicted inside-outside assembly of T3S systems from *Salmonella* spp., it was previously reported that the localization of the OM secretin EscC of the translocation-associated T3S system from EPEC depends on the ATPase EscN and the IM protein EscV. In the absence of EscV or EscN, EscC accumulates in the periplasm, suggesting that the OM localization of the secretin depends not only on the Sec pathway but also on the assembly of IM-associated components of the T3S system (196). This hypothesis is further supported by a recent publication on the mechanisms underlying the assembly of the translocation-associated T3S system from *Yersinia* spp. While the formation of the T3S system in *Yersinia* spp. was earlier proposed to be initiated by the insertion of the OM secretin (142), experimental evidence now suggests the existence of two independent assembly pathways. One assembly platform involves the insertion of the secretin into the OM followed by the assembly of the YscD and YscJ rings. The second assembly platform probably consists of members of the

TABLE 3 Characteristics of T3S-associated LTs from animal- and plant-pathogenic bacteria

LT	Organism	Characteristics or contribution to T3S and/or virulence	Reference(s)
IpgF	*S. flexneri*	No effect on virulence; LT activity demonstrated	9, 625
IagB	*S. enterica*	No effect on virulence; LT activity demonstrated	529, 625
l0045	Enterohemorrhagic *E. coli* (EHEC)	Contributes to T3S and expression of the filament protein EspA; weakly expressed; localizes mainly to the periplasm	624
EtgA	EPEC	Contributes to T3S and bacterial hemolytic activity; localizes to the periplasm; N-terminally processed; degrades peptidoglycan	192
rOrf3	*Citrobacter rodentium*	Contributes to virulence and T3S	138
HpaH	*X. campestris* pv. vesicatoria	Contributes to virulence and T3S; specifically promotes secretion and translocation of selected effector proteins	75
	Xanthomonas axonopodis pv. glycines	Contributes to virulence and HR induction; weakly expressed	272
Hpa2	*X. oryzae* pv. oryzae	(No) influence on virulence (contradictory data are published); lyses the bacterial cell wall	628, 639
	X. oryzae pv. oryzicola	Contributes to virulence and translocation of effector proteins; interacts with the translocon protein HrpF; secreted by the T3S system	319
HrpH	*P. syringae*	Contributes to effector protein translocation; overexpression in *E. coli* leads to an arrest of bacterial growth; suppresses basal plant defense responses; secreted and translocated by the T3S system	412
HopP1	*P. syringae*	Might contribute to effector protein translocation; suppresses basal plant defense responses; secreted and translocated by the T3S system	412
HopAJ1	*P. syringae*	Might contribute to effector protein translocation	412

export apparatus, including YscR, YscS, and YscT, that are required for the subsequent assembly of YscV (143). The export apparatus and the basal body are probably later joined together by the periplasmic YscJ protein (see above and Tables 1 and 2), which can directly bind to the export apparatus (143). It remains to be investigated whether a two step-assembly process is also applicable to the formation of translocation-associated and flagellar T3S systems of other bacterial species.

Contribution of Peptidoglycan-Degrading Enzymes

Macromolecular transport systems such as flagellar and translocation-associated T3S systems often require peptidoglycan-degrading enzymes, including lytic transglycosylases (LTs; also referred to as "specialized LTs"), for their efficient assembly because the natural pores of peptidoglycan are too narrow to allow the formation of these complex secretion systems (Table 3) (625; reviewed in references 282, 486, and 487). LTs are usually small proteins (150 to 250 amino acids) that cleave the beta-1,4-glycosidic bond between *N*-acetylglucosamine and *N*-acetylmuramic acid of peptidoglycan. Since LTs are ubiquitous in most peptidoglycan-containing eubacteria, they provide a potential target for new antibacterial drugs. LTs can be associated physically with components of protein secretion systems, as shown for VirB1 of the type IV secretion systems of *Agrobacterium tumefaciens* and *Brucella suis* (145, 586). This might ensure that peptidoglycan is degraded only locally.

To date, the contribution of predicted LTs to T3S and/or pathogenicity has been studied in both animal- and plant-pathogenic bacteria (75, 192, 412, 413, 624, 625, 628) (summarized in Table 3). Notably, it was observed that single LTs do not contribute significantly to T3S and virulence, presumably due to functional redundancies. Some predicted LTs that are involved in T3S, including Hpa2 from the plant-pathogenic bacterium *Xanthomonas oryzae* pv. oryzicola as well as HrpH and HopP1 from *P. syringae*, are themselves secreted, possibly to prevent further LT-mediated peptidoglycan degradation after the assembly of the secretion apparatus (319, 412) (Table 3). Interestingly, Hpa2 from *X. oryzae* pv. oryzicola contributes to effector protein secretion and interacts with the translocon protein HrpF, suggesting that it not only acts as an LT but also plays a role at the host-pathogen interface (319). The predicted LTs HrpH and HopP1 from *P. syringae* are even translocated by the T3S system into the plant cell and were shown to suppress basal plant defense responses in *Nicotiana benthamiana* (412, 413). Given the finding that peptidoglycan from animal-pathogenic bacteria is transported into the host cell, where it can be recognized by so-called pattern recognition receptors and cytoplasmic NOD proteins, it is tempting to speculate that translocated bacterial LTs might prevent the recognition of peptidoglycan by the host immune system (202, 203, 554, 571). Thus, it is possible that T3S-associated LTs have a dual activity as periplasmic proteins to promote the assembly of the T3S system and outside the bacterium after the assembly process.

RECOGNITION OF SECRETED PROTEINS

T3S Signals Are Not Conserved and Interchangeable among T3S Substrates

Substrates of T3S systems are targeted to the secretion system by a signal that is usually located within the N-terminal 20 to 30 amino acids (367, 485, 511). Although the N-terminal regions of T3S substrates are not conserved at the amino acid level, they often share specific amino acid compositions or patterns (20, 210, 336, 437, 477, 484). Furthermore, the analysis of several effector proteins from animal-pathogenic bacteria suggests that the region which harbors the N-terminal T3S signal is structurally disordered, i.e., lacks a unique tertiary structure. Intrinsically disordered protein regions can undergo structural alterations upon binding to their cognate folded partners, as was shown for the effector protein YopE from *Yersinia* spp., which binds to the cognate T3S chaperone SycE (459) (see below). The structural flexibility provided by the disordered protein regions that harbor the T3S signal might facilitate the recognition of effector proteins by components of the T3S system, including the cytoplasmic ATPase, the predicted C ring, or the cytoplasmic domains of members of the YscU and YscV protein families (Table 2; see above) (65).

The presence of an N-terminal T3S signal is not strictly conserved—although it is frequently observed—in all T3S substrates. For example, a T3S signal has been identified in the C-terminal region of the T3S effector Tir from EPEC (12). Central or C-terminal regions of T3S substrates were also shown to contribute to the secretion of the effector protein SipB from *Salmonella* spp. and the translocon protein EspB from enterohemorrhagic *E. coli* (93, 271). As an alternative to the amino acid-based T3S signal, a signal in the corresponding mRNAs of several effector proteins from *Yersinia* was proposed, suggesting a cotranslational secretion of these proteins (16, 17). However, mRNA-based T3S signals probably do not account for the high secretion rates observed for T3S substrates from animal-pathogenic bacteria. Real-time analysis of effector protein translocation revealed transport of several thousand effector protein molecules within the first few minutes of the infection process (156, 371, 488, 566, 603).

Interestingly, experimental evidence suggests that T3S signals are interchangeable (14, 63, 188, 466, 469), even between substrates of flagellar and translocation-associated T3S systems (151, 312, 321, 365, 366, 531, 587, 617). This suggests that the mechanisms underlying substrate recognition are conserved in both systems. Pathway specificity during T3S is probably conferred by the N-terminal or central region of T3S substrates, which provides the binding sites for specific T3S chaperones (see below). In this context, it is interesting that translocation-associated T3S systems can secrete and also translocate flagellin into the eukaryotic cell cytosol, where it might be recognized by the host immune system and can induce defense responses (365, 531). Recently, an interaction between flagellin and eukaryotic Nod-like receptors was demonstrated (278, 634). Experimental evidence reported for the animal-pathogenic bacterium *P. aeruginosa* revealed that the translocation-associated T3S system suppresses the expression of genes that encode components of the flagellar T3S system and *vice versa* (513). Similarly, the flagellar regulators FlhDC and FliA appear to repress the expression of *ysc* genes from *Yersinia* spp. (43, 232). The alternative sigma factor FliA is also required for the temperature-regulated expression of *ysc* genes, which are preferentially expressed at 37°C, while flagellar T3S gene expression is activated at temperatures below 30°C (104, 262, 263, 302, 462). These findings suggest an antagonistic expression of flagellar and translocation-associated T3S systems. The downregulation of flagellar T3S systems under conditions that lead to the activation of translocation-associated T3S systems, i.e., upon host cell contact, might be required to minimize host defense responses that are activated in response to flagellin.

Crossing the Borders—Translocation of Effector Proteins

The lack of amino acid sequence similarities of T3S signals significantly hampered the identification of effector proteins in plant- and animal-pathogenic bacteria. Several computational approaches that use machine-learning programs were therefore developed to identify T3S substrates from both plant- and animal-pathogenic bacteria, based on common features such as N-terminal amino acid biases in T3S signal sequences and structural elements (20, 336, 477). Additional characteristics used for the identification of effector proteins include homologies to already known effectors, the presence of typical eukaryotic protein motifs, the neighborhood of T3S chaperone genes, specific promoter elements that allow coexpression with the T3S system, and a low G+C content, which is indicative of horizontal gene transfer. Together, these approaches have led to the identification of novel effector proteins in both plant- and animal-pathogenic bacteria.

In many cases, the translocation of effector proteins into the eukaryotic cell cytosol was confirmed experimentally by the use of suitable reporter assays. For this purpose, fusion proteins between N-terminal regions of effectors and reporter proteins, such as the adenylate cyclase domain (CyaA) of the adenylate cyclase toxin of *Bordetella pertussis*, the TEM β-lactamase, or derivatives of avirulence proteins from plant-pathogenic bacteria that induce a cell death reaction inside resistant plant cells, were generated. CyaA is specifically activated in the presence of calmodulin in eukaryotic cells. The translocation of CyaA fusion proteins can therefore be determined by the measurement of intracellular cyclic AMP (cAMP) levels (485, 511, 512). In contrast to CyaA, the TEM β-lactamase cleaves the fluorescent substrate CCF2 and thus leads to a shift in the fluorescence spectrum, which can be detected in living cells (85, 345). In the last 6 years, additional assays, based on split-green fluorescent protein (split-GFP) systems, the recruitment of translocated effectors by GFP-chaperone fusion proteins inside the host cell, or the detection of translocated tetracysteine-tagged effectors by a specific fluorescing FlAsH reagent, have been developed and have allowed real-time imaging of effector protein arrival in the host cell (152, 156, 157, 488, 505, 567).

These assays revealed that in addition to the N-terminal T3S signal, translocation depends on a second protein region that is usually located within the N-terminal 50 to 100 amino acids of effector proteins and provides the binding site for a cognate T3S chaperone (52, 485, 512, 604) (see below). Furthermore, it was observed that effector protein translocation in animal-pathogenic bacteria starts within seconds after host cell contact and that the translocation kinetics of effector proteins can vary (156, 371, 488, 566, 603). Different translocation rates are indicative of a hierarchy in effector protein translocation that might guarantee the efficient manipulation of host cellular pathways by effector proteins. Furthermore, a temporal regulation of effector protein translocation might prevent an interference of effector proteins with antagonistic activities, as shown for SopE, SopE2, SipA, and SptP from *Salmonella* spp. SopE, SopE2, and SipA trigger actin polymerization, whereas SptP disrupts the changes in the actin cytoskeleton 1 to 2 h after infection (190, 521, 635, 636). While the translocation kinetics of these effector proteins differ during the initial stages of the infection process (566, 603), their cellular levels at later time points might also be regulated by other mechanisms, such as a differential proteasome-mediated degradation. It was shown that SopE and SptP are both present in the host cell about 15 min after infection and that SopE is rapidly degraded afterwards by the host cell proteasome. In contrast, SptP remains stable (290). Interestingly, the different sensitivities of SopE and SptP toward proteasomal degradation appear to depend on the N-terminal secretion and translocation signals of both proteins. Thus, a hybrid protein consisting of the N-terminal domain of SopE and the effector domain of SptP is rapidly degraded, while a fusion between the effector domain of SopE and the N-terminal region of SptP has an increased half-life (290). The precise molecular mechanisms that underlie the proteasome-dependent degradation of SopE and SptP remain to be elucidated.

Protein translocation by the T3S system has long been assumed to be a one-step transport process that is specific for effector proteins. However, a recent study reported translocation of effector

proteins from *Yersinia* spp. that localized to the bacterial cell surface prior to translocation. Translocation was dependent on the translocon, suggesting that secretion and translocation of effector proteins can be uncoupled (5). The translocation of surface-localized YopH from *Yersinia* spp. was independent of the N-terminal T3S signal (amino acids 1 to 18) but required the presence of a translocation signal within amino acids 18 to 49 (5). Interestingly, translocation has been observed not only for surface-localized effector proteins but also for extracellular proteins that are usually not transported by the T3S system. Thus, the autotransporter EspC from EPEC, which is secreted by the type V secretion system, can be translocated into eukaryotic cells in a type III secretion-dependent manner (573). The corresponding targeting signal in EspC is unknown. Taken together, these findings suggest that the mechanisms underlying translocation and recognition of translocated proteins by the translocon are more complex than initially anticipated. Future experiments will have to clarify how translocation signals target effector proteins across the host plasma membrane.

Guides and Bodyguards—the T3S Chaperones

Role of T3S chaperones in T3S substrate targeting. In addition to the secretion and translocation signal, several secreted proteins depend on cytoplasmic T3S chaperones for their efficient secretion (589, 590) (summarized in Table 4). T3S chaperones often interact as homo- or heterodimers with their cognate substrates and presumably promote the recognition of secreted proteins by components of the T3S system. Furthermore, binding of the chaperone can also prevent the premature degradation of T3S substrates. While most known T3S chaperones are cytoplasmic proteins, Spa15 from *S. flexneri* was shown to be secreted and translocated by the T3S system, suggesting that it has a second activity inside the host cell (171).

Depending on their substrate specificities, T3S chaperones have been categorized into different classes: class IA chaperones are specific for one or several homologous effector proteins, while class IB chaperones bind to different effectors with unrelated sequences (431). Known class IB chaperones include Spa15 from *S. flexneri*, InvB from *Salmonella* spp., CesT from EPEC, and HpaB from *X. campestris* pv. vesicatoria (71, 73, 149, 150, 311, 423, 546). Class II chaperones interact with translocon proteins (589), whereas chaperones of flagellar T3S systems are referred to as class III chaperones (431). Chaperones of translocon proteins usually contain tandem tetratricopeptide repeats (TPRs), which are imperfect 34-amino-acid repeats that are also present in eukaryotic chaperones and are often involved in protein-protein interactions (57, 249, 427). TPRs were also identified in the T3S chaperone YscG from *Yersinia* spp., which binds together with its cochaperone YscE to the needle protein YscF (530) (Table 4). Similarly, TPRs are present in the T3S chaperones PscG and AscG, from *P. aeruginosa* and *Aeromonas hydrophila*, respectively, which form heterotrimeric PscG-PscE-PscF and AscG-AscE-AscF complexes (86, 447, 448).

It is assumed that T3S chaperones facilitate the binding of their cognate interaction partners to components of the secretion apparatus at the IM, such as the ATPase (see above). They might thus increase the local concentrations of secretion substrates at the base of the secretion apparatus and promote their transport to the T3S system (452). As described for *S. flexneri*, the inner channels of the ATPase and the secretion apparatus, as well as the surfaces of effector proteins, have an electronegative potential which creates a repulsive force and thus a need for energy to transport secreted proteins into the T3S system (452).

CBDs in T3S substrates. Despite their moderate amino acid sequence similarities, several class I chaperones share a conserved mixed α/β fold and form dimeric structures, as revealed by crystal structure analyses (36, 37, 68, 169, 321, 327, 340, 441, 494, 520, 553, 565). Conserved structural features were also described for the chaperone-binding domains (CBDs) of T3S substrates, which are often located within the N-terminal 50 to 100 amino acids and are wrapped around the chaperone dimer in an extended conformation (37, 321, 441, 494, 520, 598). Chaperone-bound CBDs were therefore proposed to serve as three-dimensional targeting signals that are recognized by components of the T3S system at the IM (37, 167, 321). In line with this model are the findings that the CBD is often required for efficient translocation (Table 4) and that binding of the chaperone SycE to the effector protein YopE from *Yersinia* spp. induces a disorder-to-order transition in the CBD of YopE (459). Interestingly, it was shown that single amino acid substitutions in the CBD of YopE result in reduced translocation of YopE but do not affect YopE secretion or the interaction of YopE with SycE (460). This suggests that a postulated three-dimensional targeting signal in the CBD is required for the efficient translocation of effector proteins. Notably, however, it is not yet known whether T3S systems harbor specific recognition sites for translocated proteins. Given the finding that most T3S chaperones are not secreted and that mutations in the CBD specifically affect translocation but not secretion of YopE, it is possible that the specific targeting of T3S substrates to the translocon is controlled in the bacterial cytoplasm. The molecular mechanisms underlying the potential cross talk between the translocon and components of the T3S system in the IM remain to be investigated.

Notably, the N-terminal protein region including the CBD is not always sufficient to target T3S substrates for efficient secretion by the translocation-associated T3S systems. This was shown, for instance, for the T3S effectors SipB and Tir, from *Salmonella* spp. and EPEC, respectively, which depend on C-terminal protein regions for efficient secretion (12, 271) (see above). Furthermore, secretion and/or translocation of some effector proteins was also observed in the absence of the CBD, suggesting that the CBD *per se* is not always essential for protein export (52, 151, 314, 321, 556, 604). However, in many cases it was not analyzed whether the observed secretion of effector derivatives deprived of their CBDs was still mediated by the translocation-associated T3S system. As shown for the effector protein SptP from *Salmonella*, the absence of the CBD can lead to a loss of pathway specificity and thus to a secretion of SptP by the flagellar T3S system (312). It was therefore proposed that the binding of T3S chaperones to the CBD confers the specific secretion of proteins by the translocation-associated T3S system (312). However, a later study revealed that it is the CBD itself which determines the recognition specificity of flagellar or translocation-associated T3S systems (151).

In addition to their roles in protein export, CBDs might also provide a membrane-targeting signal, as shown for the CBD of the effector protein YopO from *Yersinia* spp. Binding of the chaperone SycO to the CBD of YopO could prevent the membrane localization of YopO inside the bacterium. In agreement with this model, deletion of the CBD in YopO did not interfere with the secretion and translocation of the protein by the wild-type strain but abolished membrane localization of YopO inside the host cell

TABLE 4 Known T3S chaperones from selected animal- and plant-pathogenic bacteria

Organisms and chaperone[a]	Interaction partner(s)[b]	Location of CBD[c]	Description/comments	Reference(s)
Yersinia spp.				
SycD (LcrH) (*Ysc T3SS*)	YopB (T), YopD (T), YscY (C), YscM1 (R), YscM2 (R)	aa 53 to 149 and 278 to 292 of YopD; no discrete CBD in YopB	Dimerizes; contains three TPRs; stabilizes YopB and YopD; regulatory function (see Fig. 7)	69, 146, 147, 180, 181, 404, 489, 537
SycE (*Ysc T3SS*)	YopE (E), YscM1 (R), YscM2 (R)	aa 15 to 50 of YopE	Dimerizes; promotes a disorder-to-order transition in the CBD of YopE; alanine substitutions in the CBD of YopE do not affect SycE binding and YopE secretion but lead to severely reduced translocation of YopE	36, 37, 90, 169, 459, 460, 485, 489, 537, 604
SycH (*Ysc T3SS*)	YopH (E), YscM1 (R), YscM2 (R)	aa 20 to 69 of YopH; N-terminal region of YscM2	Dimerizes; YscM1 and YscM2 share aa similarity with the CBD of YopH; regulatory function (see Fig. 7)	80, 168, 441, 604
SycN, YscB (*Ysc T3SS*)	YopN (R/E)	aa 30 to 76 of YopN	SycN and YscB form a heterodimer and stabilize YopN	126, 244, 494
SycO (*Ysc T3SS*)	YopO (E), YscM1 (R)	aa 20 to 77 of YopO	Dimerizes; masks membrane localization domain of YopO; overproduction of SycO leads to reduced Yop secretion	144, 314
SycT (*Ysc T3SS*)	YopT (E)	At least aa 52 to 103 of YopT	Dimerizes; binding to catalytically inactive YopT(C139S) is reduced	68, 240, 327
YscE, YscG (*Ysc T3SS*)	YscF (N)	C-terminal region of YscF	YscE and YscG form a heterodimer; YscG contains TPRs and shares a similar fold with LcrH; YscE shares structural similarity with the needle protein MxiH from *S. flexneri*	125, 440, 530
YscY (*Ysc T3SS*)	YscX (EC)	aa 50 to 110 of YscX	Regulatory function (see Fig. 7)	127, 241
SycP (*Ysa T3SS*)	YspP (E)		Dimerizes; stabilizes YspP	352
SycB (*Ysa T3SS*)	YspB (T), YspC (T)		Dimerizes; stabilizes YspB; together with the AraC-type regulator YscE regulates the expression of *ysa* T3S genes	28, 178, 581
Shigella spp.				
IpgA	IcsB (E)	aa 171 to 247 of IcsB	Stabilizes IcsB; *icsB* and *ipgA* can be translated as a single fusion protein	409
IpgC	IpaB (T), IpaC (T), MxiE (R)	aa 15 to 45 and 48 to 74 of IpaB; aa 50 to 80 (213), 73 to 122 (422) and/or 33 to 73 (328) of IpaC	Dimerizes; contains TPR motifs; stabilizes IpaB and IpaC; probably acts as a coactivator of the AraC-type transcriptional activator MxiE (see Fig. 7)	27, 35, 213, 328, 338, 354, 362, 422, 443
IpgE	IpgD (E)		Stabilizes IpgD	405
Spa15	IpaA(E), IpgB1 (E), OspC3 (E), OspB (E), OspD1 (R)	aa 26 to 141 of OspC3; aa 263 to 365 of IpaA; aa 23 to 190 of IpgB1	Dimerizes; is secreted; stabilizes IpgB but not IpaA; binds to the secreted antiactivator OspD1 and acts as coantiactivator (see Fig. 7)	171, 422, 423, 565
IpaD	IpaD (T)		Possesses self-chaperoning activity	252
Salmonella spp.				
InvB (*SPI-1*)	SipA (SspA) (E), SopA (SipF) (E), SopE (E), SopE2 (E)	aa 1 to 45 of SopA; aa 1 to 158 of SipA; aa 30 to 45 of SopE	Dimerizes; contributes to stability of SopE2 and SipA; CBD of SopA is required for translocation; CBD of SopE prevents secretion by SPI-1 or flagellum in the absence of InvB	58, 149-151, 219, 311, 321
SicA (*SPI-1*)	SipB (T), SipC (T), InvF (R)	aa 80 to 100 of SipB	Self-interacts; coactivator of the AraC-type transcriptional regulator InvF; stabilizes SipB and SipC; SipB is stable and secreted in a *sicA sipC* mutant; CBD of SipB is not sufficient to target the protein to the translocation-associated T3SS	119, 120, 271, 559
SicP (*SPI-1*)	SptP (E)	aa 35 to 139 of SptP	Dimerizes; stabilizes SptP; translation of SicP is required for translation of SptP (translational coupling)	76, 189, 520
SigE (*SPI-1*)	SopB (SigD) (E)		Dimerizes; stabilizes SopB	121, 231, 275, 340
SrcA (*SPI-2*)	SseL (E), PipB2 (E)		Dimerizes; multicargo T3S chaperone; the *srcA* gene is unlinked to the T3S system genomic region	101
SscB (*SPI-2*)	SseF (E)		Stabilizes SseF	115
SseA (*SPI-2*)	SseB (T), SseD (T)	aa 147 to 169 of SseB; aa 138 to 194 of SseD (but aa 32 to 82 of SseD also contribute to the binding of SseA)	Contributes to stability of SseB	99, 472, 641, 642
SsaE (*SPI-2*)	SseB (T)		Also contributes to secretion of the effector PipB	369
$SsaQ_S$ (*SPI-2*)	$SsaQ_L$ (YscQ homolog)		Generated by tandem translation of $ssaQ_L$	622

(Continued on following page)

TABLE 4 (Continued)

Organisms and chaperone[a]	Interaction partner(s)[b]	Location of CBD[c]	Description/comments	Reference(s)
EPEC/EHEC				
CesA2 (L0017)	EspA (F)		Inhibits polymerization of EspA; stabilizes EspA	527
CesAB (CesA)	EspA (F), EspB (T)		Dimerizes; stabilizes EspA	111, 613
CesD	EspD (T)		Also contributes to secretion of EspB	580
CesD2	EspD (T)		Stabilizes EspD	402
CesF	EspF (E)			154, 575
CesL	SepL (E)			620
CesT	Tir (E), Map (E), NleA (E), EspF (E), EspG (E), EspZ (E), NleG (E), NleH (E), NleH2 (E)	N-terminal 50 to 100 aa of Tir	Dimerizes; contributes to stability of Map; also contributes to secretion of effector NleI	1, 109, 134, 153, 318, 340, 545, 546
P. syringae				
HrpG	Unknown		Acts as suppressor of the negative regulator HrpV (see Fig. 7)	593
ShcA	HopPsyA (HopA1) (E)	N-terminal 166 aa of HopPsyA		564
ShcM	HopPtoM (HopM1) (E)	aa 100 to 400 of HopPtoM	Protects HopPtoM from Lon-mediated degradation	22, 333
ShcF	HopPtoF (HopF2) (E)		Stabilizes HopPtoF	499
ShcV	HopPtoV (HopV1) (E)	aa 76 to 125 of HopPtoV		592
ShcO1	HopO1-1 (E), HopS1 (E), HopS2 (E)	Central part of HopO1-1	Homologous to ShcS1; can interact with ShcS1	209, 259
ShcS1	HopO1-1 (E), HopS1 (E), HopS2 (E)	Central part of HopO1-1	Can interact with ShcO1; forms homodimers	209, 259
ShcS2	HopO1-1 (E), HopS1 (E), HopS2 (E)	Central part of HopO1-1	Homologous to ShcS1	209
X. campestris pv. vesicatoria				
HpaB	AvrBs1 (E), AvrBs3 (E), HpaA (E), HpaC (R)	aa 1 to 50 of AvrBs3; aa 225 to 275 of HpaA	Essential for pathogenicity; contributes to the efficient T3S and translocation of multiple effector proteins	71, 73, 331
Erwinia amylovora				
DspB/F	DspA/E (E)	aa 51 to 100 and C-terminal region of DspA/E	N-terminal CBD is required for DspA/E translocation (411); minimal translocation signal does not comprise CBD (556)	193, 411, 556

[a] T3S chaperones of translocation-associated T3S systems. For pathogens with more than one T3S system, the type of the respective T3S system is written in italics. Alternative protein names are given in parentheses. T3SS, T3S system.
[b] Known interaction partners are categorized into effectors (E), translocon proteins (T), needle proteins (N), filament proteins (F), regulators (R), chaperones (C), and extracellular proteins of the T3S system (EC). Alternative protein names are given in parentheses.
[c] aa, amino acids.

(314). Similar to SycO, the T3S chaperones SycE and SycT might mask a membrane localization domain in their corresponding interaction partners, i.e., YopE and YopT (287, 314). These observations suggest that the CBD of effector proteins not only is essential for translocation *per se* but also could exert an inhibitory influence on translocation by promoting membrane localization of the effector protein inside the bacterium. Taken together, these findings imply that T3S chaperones fulfill multiple functions that can vary in different pathogens. Thus, T3S chaperones not only promote stability and secretion of their cognate interaction partners but also could prevent membrane localization of effector proteins inside the bacterial cytosol. Furthermore, experimental evidence suggests that several T3S chaperones can be involved in the regulation of T3S gene expression (see below). Given that T3S chaperones often do not share significant sequence similarities with each other, the term "T3S chaperone" therefore refers to a rather heterogeneous group of proteins with structural similarities but various functions.

Contribution of T3S chaperones to the establishment of a secretion hierarchy. In addition to their contribution to the docking and recognition of T3S substrates, T3S chaperones might also impose a hierarchy on the translocation of effector proteins. It was shown that deletion of the CBD in YopE abolishes the translocation of YopE by the wild-type strain but not by a polyeffector mutant, suggesting that the binding of the chaperone helps the effector to compete with other effectors for translocation (52). A chaperone-dependent hierarchy in effector protein translocation was also observed in EPEC. In this case, the class IB chaperone CesT from EPEC promoted the secretion of the effector protein Tir, which is itself required for the efficient secretion of additional CesT-dependent effectors. In the absence of Tir, effector protein secretion was severely reduced, presumably because of enhanced levels of free CesT that blocked the T3S system (545) (Fig. 5A). It was proposed that CesT regulates effector protein secretion after being released from Tir, presumably by binding to components of the T3S system at the IM. A negative regulation of effector protein secretion by a potentially uncomplexed chaperone was also observed for the class IB chaperone HpaB from *X. campestris* pv. vesicatoria, which interacts with the effector protein HpaA. Secretion of HpaA is probably required to liberate HpaB and thus to allow the efficient HpaB-mediated secretion and translocation of additional effector proteins. In the absence of HpaA, increased

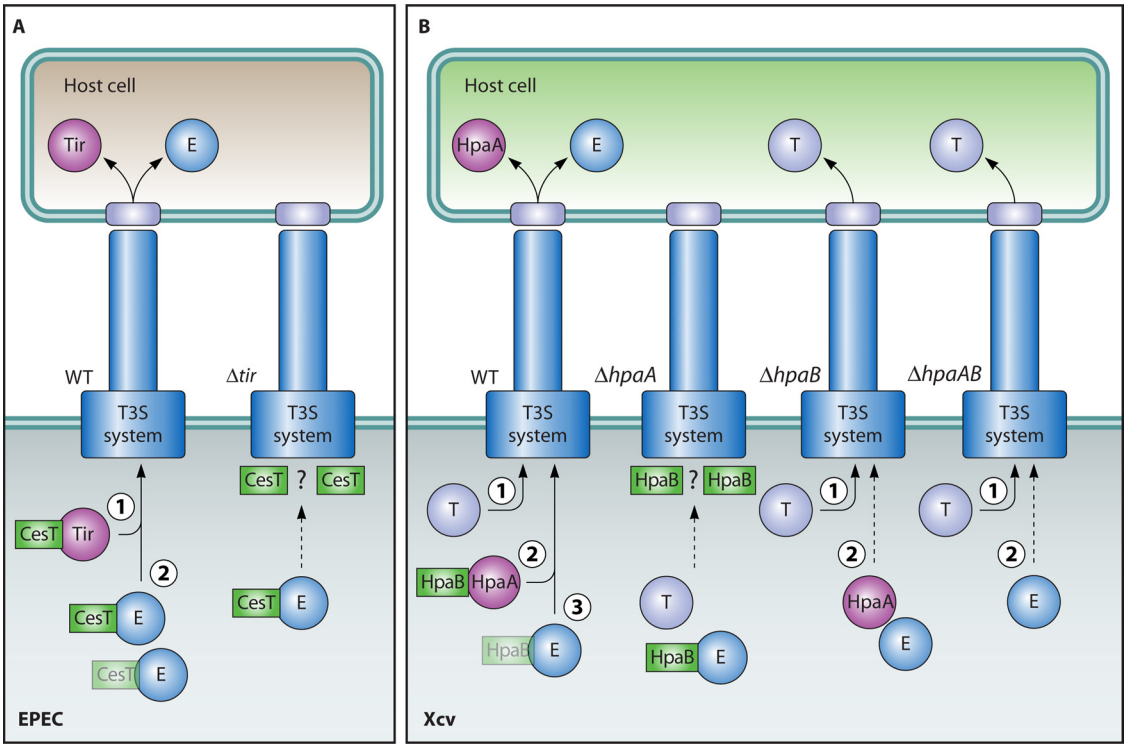


FIG 5 Predicted functions of class IB chaperones during control of effector protein secretion in EPEC and *X. campestris* pv. vesicatoria. (A) Model for the function of the class IB T3S chaperone CesT from EPEC. CesT promotes the secretion and translocation of Tir, which is the first effector protein (E) that is translocated into the host cell. It is assumed that CesT binds preferentially to Tir to promote Tir secretion after assembly of the T3S system. In the absence of Tir, uncomplexed CesT might block the secretion of effector proteins by the T3S system. The question mark indicates that it is still unknown whether the inhibitory activity of CesT is linked to its potential association with components of the T3S system. Dashed arrows indicate reduced secretion and/or translocation rates. (B) Hypothetical mode of action of the class IB T3S chaperone HpaB from *X. campestris* pv. vesicatoria. HpaB binds to and promotes the secretion of the effector protein HpaA and additional effector proteins (E) after the secretion of translocon proteins (T). Similar to Tir, HpaA is presumably the first effector protein that is bound to the chaperone and travels the T3S system. In the absence of HpaA, the efficient secretion of effector, pilus, and translocon proteins is suppressed, probably by uncomplexed HpaB that binds to components of the secretion apparatus. The question mark indicates that it is unknown whether the association of HpaB with the T3S system contributes to its inhibitory activity. Interestingly, in the absence of HpaB, translocon proteins are secreted and even translocated, suggesting that they contain a functional translocation signal that is suppressed by HpaB. Translocon proteins are also efficiently secreted in an *hpaAB* double deletion mutant, in which T3S is not suppressed by uncomplexed HpaB.

amounts of free HpaB lead to reduced T3S of pilus, translocon, and effector proteins (331) (Fig. 5B). The additional deletion of *hpaB* in an *hpaA* mutant therefore restores the efficient secretion of pilus and translocon proteins (331) (Fig. 5B). Notably, HpaB not only imposes a hierarchy on effector protein translocation but also appears to prevent the translocation of extracellular components of the T3S system, such as components of the translocon (Fig. 5B). Thus, it was shown that the N-terminal regions of translocon and pilus proteins can target a reporter protein for translocation in an *hpaB* mutant but not in the wild-type strain. This suggests that pilus and translocon proteins harbor a translocation signal that is suppressed during HpaB-mediated effector protein translocation (71; my unpublished data).

FEEDBACK CONTROL—HOW GENE EXPRESSION IS COUPLED TO THE SECRETORY ACTIVITY OF THE T3S SYSTEM

T3S is controlled not only on the posttranslational level but also by transcriptional regulators, which often couple the expression of genes that encode components and substrates of the T3S system to the secretory activity of the system. A common regulatory principle involves the interaction of a T3S chaperone with either its cognate T3S substrate or a cytoplasmic regulatory protein. Upon activation of T3S, the chaperone is liberated from its secreted binding partner and can bind to regulatory proteins inside the cytoplasm, including transcriptional activators or antiactivators (Fig. 6A). Binding of the T3S chaperone can positively regulate transcriptional activators or counteract antiactivators that act as suppressors of transcriptional activators. The interaction of a regulatory chaperone with an antiactivator thus relieves the inhibitory effect of the antiactivator on the activity of the transcriptional activator and leads to the induction of T3S gene expression (Fig. 6B). Known transcriptional regulators that are involved in the control of T3S gene expression and corresponding co-, anti-, or antiantiactivators are summarized in Table 5 and briefly described below.

Hierarchical Control of Gene Expression in Flagellar T3S Systems

In flagellar T3S systems, genes encoding components and substrates of the secretion apparatus are not expressed simultaneously but activated at different stages of the secretion process. According to their temporal expression patterns, flagellar genes are organized into three different classes (299). Class I genes encode the transcriptional activators FlhD and FlhC, which initiate

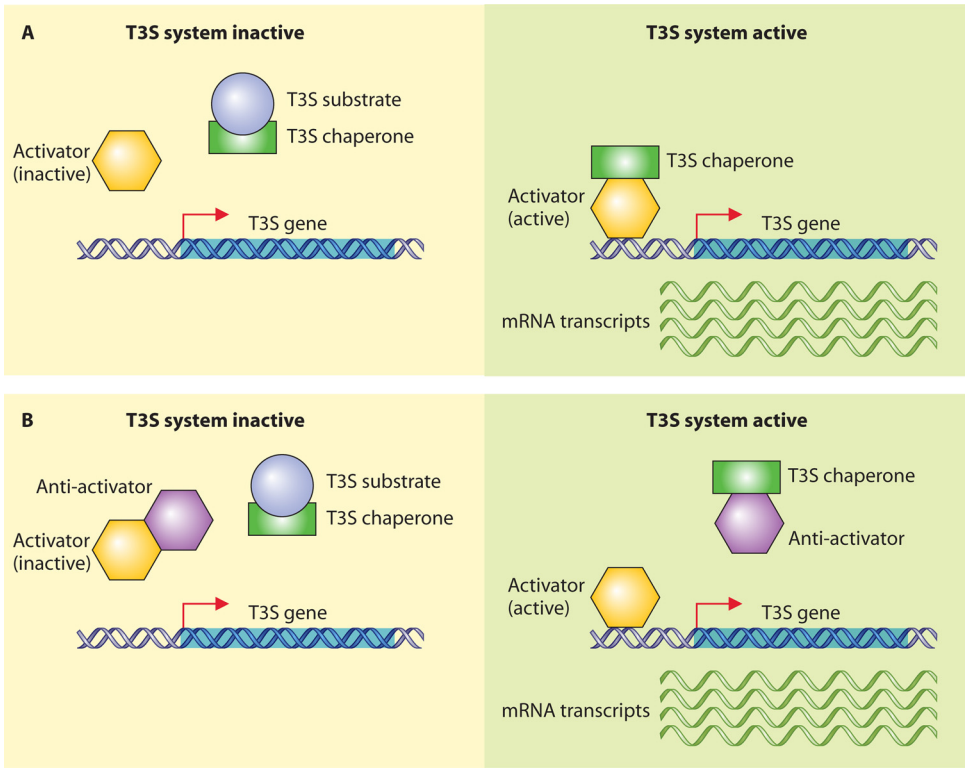


FIG 6 Control of T3S gene expression by regulatory chaperones. (A) Proposed mode of action of T3S chaperones that act as coactivators of transcriptional regulators. When the T3S system is inactive, T3S chaperones are bound by their cognate substrates in the bacterial cytosol. The activation of the T3S system leads to the secretion of T3S substrates and thus to the liberation of corresponding T3S chaperones, which can subsequently interact with transcriptional activators and promote T3S gene expression. T3S chaperones are represented by green rectangles, and T3S substrates are represented by circles. Transcriptional activators are depicted in yellow. (B) Model for the activity of T3S chaperones that act as antiantiactivators. When the T3S system is inactive, T3S chaperones are bound by their corresponding T3S substrates. The induction of T3S leads to the liberation of T3S chaperones, which can subsequently bind to an antiactivator that suppresses the activity of a transcriptional activator. Upon interaction with the T3S chaperone, however, the antiactivator is released from the transcriptional activator, and the latter can induce T3S gene expression.

the assembly of the flagellum. FlhD and FlhC activate genes that are expressed under the control of class II promoters and encode components of the MS, C, P, and L rings, the inner rod, and the extracellular hook (299, 322). The class II gene product FlgM binds to the sigma factor FliA (σ^{28}) inside the bacterial cytoplasm and thus prevents the association of FliA with the RNA polymerase (8, 81, 82, 200, 414, 415) (Fig. 7). Upon completion of the hook-basal body, FlgM is secreted and liberates FliA, which can subsequently activate class III genes that encode proteins involved in the formation of the flagellar filament and the stator complexes (234, 266, 294, 299, 323, 414) (Fig. 7). Notably, FliA acts not only as a sigma factor but also as a T3S chaperone for FlgM. Since FliA and FlgM are encoded by both class II and class III genes, they can autoregulate their own expression levels (201, 324). Translation of the *flgM* class III mRNA is enhanced by the T3S chaperone FlgN, which binds to the hook-filament junction proteins FlgK and FlgL (6, 30, 184, 264). Thus, upon secretion of FlgK and FlgL, liberated FlgN promotes the translation of FlgM (encoded by the class III *flgM* gene), which can bind to and inhibit FliA (Fig. 7).

In addition to FlgN, expression of class II genes can also be repressed by FliT, which acts as a T3S chaperone of the filament cap protein FliD and also binds to the regulatory FlhDC proteins (184, 297, 610). Secretion of FliD after hook formation liberates FliT, which subsequently binds to FlhC and thus suppresses the FlhDC-dependent activation of class II gene expression (30, 610) (Fig. 7). FliT also interferes with the autoinhibitory activity of FlhDC on their own expression and thus restores the expression of class I genes (295, 610).

Control of *yop* Gene Expression in *Yersinia* spp.

The coupling of transcriptional gene regulation and T3S has been described not only for flagellar T3S systems but also for translocation-associated T3S systems from animal-pathogenic bacteria. In *Yersinia* spp., the expression of effector (*yop*) genes is specifically activated after host cell contact upon secretion of the translocon protein YopD, which acts a negative regulator of *yop* gene expression inside the bacterial cytoplasm. YopD presumably binds to the 5′-untranslated regions of *yop* mRNAs in complex with its chaperone, LcrH (also known as SycD) (15, 181). Binding of YopD-LcrH to the *yop* mRNA might prevent the access of ribosomes and facilitate mRNA degradation, thus leading to reduced Yop levels (88) (Fig. 7). Furthermore, LcrH might have an additional regulatory role in the suppression of *yop* gene expression that is independent of its binding to YopD and involves an interaction of LcrH with YscY, a predicted chaperone of the secreted YscX protein (56, 127, 181).

A second transcriptional repressor from *Yersinia* spp. is the secreted LcrQ protein, which was identified in *Y. pseudotuberculosis*.

TABLE 5 Transcriptional and posttranscriptional control proteins that link T3S gene expression with the secretory activity of flagellar and translocation-associated T3S systems

Organism and control protein[a]	Predicted functions/characteristics[b]
Salmonella spp.	
Transcriptional control proteins (flagellar T3S system)	
FlhDC	Transcriptional activators for class II genes; form an $FlhD_4$-$FlhC_2$ complex with DNA-binding activity; promote the σ^{70}-dependent transcription of class II genes; also act as autoinhibitors of their own transcription
FlgM*	Anti-σ^{28} factor; inhibits the σ^{28}-dependent RNA polymerase that is specific for class III promoters; expressed from class II and class III promoters; class II-expressed FlgM interacts with FliA (σ^{28}); secreted upon hook-basal body completion
FliA	σ^{28} factor; associates with the RNA polymerase and induces the expression of class III genes after secretion of the anti-σ^{28} factor FlgM; expressed from class II and class III promoters; also acts as a chaperone for FlgM
FlgN	Chaperone of hook-filament junction proteins FlgK and FlgL; promotes the translation of class III-expressed *flgM*
FliT	Chaperone of filament cap protein FliD; inhibits the FlhDC-dependent activation of class II gene expression and FlhDC autoinhibition (leads to reinitiation of class I gene expression) when liberated from FliD
Posttranscriptional control proteins (translocation-associated T3S system)	
InvE (SPI-1)	YopN homolog; negative regulator of effector protein secretion; deletion of *invE* leads to reduced secretion of translocon proteins
SsaL* (SPI-2)	YopN homolog; negative regulator of effector protein secretion; deletion of *ssaL* leads to reduced secretion of translocon proteins; interacts with the SsaM-SpiC complex; dissociation of the SsaL-SsaM-SpiC complex at pH 7 activates effector protein secretion
SsaM (SPI-2)	Interacts with SpiC; a SpiC-SsaM complex interacts with SsaL at pH 7; negative regulator of effector protein secretion; deletion of *ssaM* leads to reduced secretion of translocon proteins
SpiC (SPI-2)	Interacts with SsaM; a SpiC-SsaM complex interacts with SsaL at pH 7; negative regulator of effector protein secretion; deletion of *spiC* leads to reduced secretion of translocon proteins
Yersinia spp. (translocation-associated T3S system)	
Transcriptional control proteins	
YopD*	Translocon protein; negative regulator of *yop* gene expression; binds in complex with the chaperone LcrH to the 5′-untranslated region of *yop* mRNAs
LcrH	Chaperone of YopD; negative regulator of *yop* gene expression; regulatory activity also requires interaction with the predicted T3S chaperone YscY
LcrQ*	Transcriptional repressor; blocks *yop* gene expression in complex with the chaperone SycH; regulatory activity depends on YopD
SycH	Chaperone of LcrQ and YopH; promotes YopH secretion after export of LcrQ
Posttranscriptional control proteins	
YopN*	Negative regulator of Yop and translocon protein secretion; regulatory activity depends on interaction with TyeA, SycN, and YscB; a YopN-TyeA complex acts as an internal plug of the T3S system; secretion of YopN under T3S-permissive conditions relieves the negative regulatory effect
TyeA*	Interacts with and prevents secretion of YopN
SycN	Chaperone of YopN
YscB	Chaperone of YopN
LcrG	Negative regulator of Yop secretion; inhibitory influence is counteracted by binding of LcrV; LcrG also promotes the secretion of LcrV
LcrV*	Tip complex protein; interacts with LcrG and counteracts the LcrG-mediated repression of Yop secretion
P. aeruginosa (translocation-associated T3S system)	
Transcriptional control proteins	
ExsA	AraC-type transcriptional activator; interacts with the antiactivator ExsD under T3S-nonpermissive conditions
ExsD	Antiactivator of ExsA; binds to ExsA and inhibits ExsA-dependent transcription
ExsC	Antiantiactivator; binds to ExsD and counteracts the negative regulatory activity of ExsD; also acts as a T3S chaperone of ExsE; secretion of ExsE liberates ExsC and allows its interaction with ExsD
ExsE*	Inhibitor of ExsC
Posttranscriptional control proteins	
PopN*	Blocks T3S of effector proteins from inside the cytosol similarly to YopN; does not interfere with secretion of translocon proteins
PcrG	Internal plug of T3S system; interacts with PcrV; inhibitory activity of PcrG is independent of its interaction with PcrV
PcrV*	Tip complex protein; interacts with PcrG; presumably acts as external plug of the T3S system
S. flexneri (translocation-associated T3S system)	
Transcriptional control proteins	
MxiE	AraC-type transcriptional activator; required for effector gene expression; activity of MxiE depends on its interaction with IpgC
IpgC	Coactivator of MxiE; interacts with MxiE and OspD1; chaperone of the translocon proteins IpaB and IpaC; binding of IpaB and IpaC to IpgC inhibits MxiE/IpgC-dependent activation of effector gene expression
OspD1*	Antiactivator of MxiE; binds to MxiE and to the chaperone Spa15
Spa15*	Class IB T3S chaperone; promotes secretion of OspD1; coantiactivator that inhibits MxiE activity in complex with OspD1; T3S-inducing conditions lead to the secretion of OspD1 and Spa15 and thus to the activation of MxiE/IpgC-dependent effector gene expression
Posttranscriptional control proteins	
IpaB*, IpaD*	Tip complex proteins; might serve as an external plug to control effector protein secretion
MxiC*	YopN homolog; negative regulator of Yop secretion; deletion of *mxiC* leads to reduced secretion of translocon proteins in response to Congo red induction

(Continued on following page)

TABLE 5 (Continued)

Organism and control protein[a]	Predicted functions/characteristics[b]
Enterohemorrhagic *E. coli* (translocation-associated T3S system)	
Posttranscriptional control proteins	
SepL	YopN homolog; deletion of *sepL* leads to reduced secretion of translocon and enhanced secretion of effector proteins; SepL interacts with SepD, Tir, and the T3S chaperone CesL
SepD	Interacts with SepL; deletion of *sepD* leads to reduced secretion of translocon proteins and enhanced secretion of effectors

[a] Secreted proteins are indicated by asterisks, transcriptional regulators are shown in bold, and T3S chaperones are underlined.
[b] References are given in the text.

LcrQ blocks *yop* gene expression in complex with the chaperone SycH. The LcrQ homologs YscM1 and YscM2 from *Y. enterocolitica* might act in a similar manner (79, 439) (Fig. 7). Although the molecular mechanisms underlying the LcrQ/YscM-mediated control of *yop* gene expression are not yet understood, both proteins likely act on the transcriptional level (519). Secretion and translocation of LcrQ/YscM upon activation of the T3S system relieve the negative effect on *yop* gene expression and also liberate SycH, which is a common T3S chaperone of LcrQ/YscM and YopH and presumably promotes secretion and translocation of YopH after LcrQ/YscM export (78, 456, 607) (Fig. 7). LcrQ-YscM-mediated *yop* gene repression is linked to the action of YopD, but the mechanisms underlying the functional interplay between LcrQ/YscM and YopD are still unclear.

Regulation of T3S Genes in *S. flexneri*, *Salmonella* spp., and *P. aeruginosa*

Similar to the case of *Yersinia* spp., the expression of effector genes in *S. flexneri* is triggered upon host cell contact. Gene induction depends on the AraC-type transcriptional activator MxiE and its interacting coactivator, IpgC (354, 443). Under noninducing conditions, MxiE interacts with the antiactivator OspD1 and the coantiactivator Spa15, which both prevent MxiE-dependent gene expression (430) (Fig. 7). Spa15 is a class IB T3S chaperone that is itself secreted by the T3S system (171, 423, 430) (see above). Secretion of OspD1 (and Spa15) upon activation of the T3S system leads to the liberation of MxiE, which subsequently activates effector gene expression in complex with IpgC (430) (Fig. 7). IpgC also acts as a T3S chaperone that promotes the secretion of the translocon proteins IpaB and IpaC (363). Under T3S-inducing conditions, IpgC is released from its secreted interaction partners and binds to MxiE, thus promoting MxiE-dependent gene expression (430). A similar mechanism might underlie the function of the MxiE and IpgC homologs InvF and SicA, respectively, from *Salmonella* spp. (118, 120).

An AraC-type transcriptional activator (designated ExsA) that controls effector gene expression has also been described for *P. aeruginosa*. When the T3S system is inactive, ExsA is bound by the antiactivator ExsD and does not induce gene expression (355, 608) (Fig. 7). The inhibitory activity of ExsD is counteracted by the antiantiactivator ExsC, which interacts with ExsD but also acts as a T3S chaperone for the T3S substrate ExsE (122). ExsC is therefore complexed with ExsE in the cytoplasm when the T3S system is inactive (Fig. 7). Activation of the T3S system, however, results in the secretion of ExsE and thus in the liberation of ExsC, which can subsequently interact with the antiactivator ExsD. This leads to the release of ExsD-bound ExsA and to the activation of ExsA-dependent gene expression (455, 561).

Regulation of T3S Gene Expression in *P. syringae* by the Regulatory T3S Chaperone-Like Protein HrpG and the Lon Protease

In *P. syringae*, the expression of T3S genes is specifically induced when the bacterium enters the plant apoplast, by the two regulatory proteins HrpR and HrpS, which belong to the NtrC family of two-component regulators and interact with each other (54). HrpR and HrpS induce the expression of the alternative sigma factor HrpL, which binds to conserved elements (termed *hrp* [hypersensitive response and pathogenicity] boxes) in the promoter regions of T3S genes (541). The HrpL-dependent activation of T3S gene expression is counteracted by the negative regulator HrpV, which presumably acts upstream of HrpL and interacts with HrpS (446, 593) (Fig. 7). The activity of HrpV can be suppressed by the cytoplasmic HrpG protein, which shares typical features of a T3S chaperone and probably binds not only to HrpV but also to a secreted interaction partner that has not yet been identified (593). It is assumed that the activation of T3S leads to the release of HrpG from its predicted secreted interaction partner and thus to the subsequent interaction of HrpG with HrpV, which counteracts the negative effect of HrpV on T3S gene expression (Fig. 7).

In addition to HrpR, HrpS, HrpL, HrpV, and HrpG, the Lon protease was identified as another player in the control of T3S gene expression in *P. syringae*. The Lon protease is an ATP-dependent serine protease that is involved in the degradation of unstable or misfolded proteins and can contribute to the regulation of T3S genes (464, 551). In *P. syringae*, the Lon protease acts as a negative regulator by degrading HrpR, specifically under T3S-repressing conditions (54, 303, 420, 612). Notably, the Lon protease is also involved in the degradation of effector proteins from *P. syringae*. However, most effector proteins are protected from Lon-mediated degradation by the binding of their corresponding T3S chaperones (333). Lon-mediated degradation of regulatory proteins was also shown for the flagellar sigma factor FliA from *E. coli*, which can be protected from Lon-dependent degradation by interaction with the anti-sigma factor FlgM (see above) (24). Furthermore, in *Salmonella* spp., the Lon protease degrades the transcriptional activators HilC and HilD, which are involved in the regulation of the SPI-1-encoded T3S system (539). In *Yersinia* spp., the Lon protease and the ATP-dependent ClpXP protease degrade a small histone-like protein designated YmoA, which represses the expression of T3S genes (246). In conclusion, these findings suggest that the Lon protease is involved in the regulation of T3S gene expression in both plant- and animal-pathogenic bacteria.

ORCHESTRATION OF T3S—HOW SUBSTRATE SPECIFICITY IS CONTROLLED

Components of the extracellular needle or pilus are most likely the first proteins that travel the secretion apparatus and are therefore also referred to as "early" T3S substrates. In animal-pathogenic bacteria, the formation of T3S needles is a tightly controlled process that ensures a defined distribution of the lengths of needle structures, with a peak at 45 nm (*Shigella* spp. [45, 540]), 60 nm (*Yersinia* spp. [224, 257]), or 80 nm (*Salmonella* spp. [291]) (44, 257, 293, 578, 579) (Table 6 and see below). Since needles have to be sufficiently long to bridge the extracellular space between the bacterium and the host cell, needle length control is probably essential for the efficient translocation of effector proteins. In agreement with this model, a correlation between needle length and the length of the adhesin YadA from *Y. enterocolitica* has been observed (394). Shorter YadA molecules allowed effector protein translocation by short needles that would not be translocation competent in the context of the wild-type YadA protein (394). Notably, additional studies of *Shigella* and *Salmonella* spp. also revealed an influence of the length of extracellular lipopolysaccharide molecules on effector protein translocation (226, 596).

T3S4 Proteins and Their Interplay with YscU/FlhB Family Members

Since the formation of the pilus/needle is a prerequisite for T3S, it presumably precedes the secretion of intermediate (translocon proteins) and late (effector proteins) substrates, suggesting that the substrate specificity of the T3S system switches. The predicted switch in T3S substrate specificity from early to late substrates is mediated by T3S substrate specificity switch (T3S4) proteins, which have been studied intensively in animal-pathogenic bacteria. T3S4 proteins are often themselves secreted by the T3S system and are involved not only in the substrate specificity switch but also in length control of the extracellular needle (Table 6). Lack of a functional T3S4 protein usually results in increased needle length and a reduced secretion of late substrates. To date, T3S4 proteins have been described for translocation-associated T3S systems from several animal-pathogenic bacteria (YscP from *Yersinia* spp., Spa32 from *Shigella* spp., and InvJ from *Salmonella* spp.) and the plant-pathogenic bacterium *X. campestris* pv. vesicatoria (HpaC) (Table 6). Furthermore, in flagellar T3S systems, the T3S4 protein FliK has been identified, which switches the substrate specificity from early (hook components) to late (filament proteins) substrates after the hook has reached a length of approximately 55 nm (374, 386).

T3S4 proteins share little amino acid sequence identity with each other but contain a structurally conserved C-terminal domain (termed the T3S4 domain) that is probably essential for the substrate specificity switch and harbors a P-X-L-G amino acid motif (2). T3S4 proteins from both translocation-associated and flagellar T3S systems interact with the C-terminal cytoplasmic domains of members of the conserved YscU/FlhB family of IM proteins, as shown for the T3S4 proteins FliK, Spa32, and HpaC (50, 332, 381, 392) (Tables 2 and 6). Binding of T3S4 proteins to the C-terminal domains of FlhB, YscU, and homologs might induce a conformational change in these domains that alters the substrate specificity of the T3S system. This hypothesis is corroborated by the finding that single point mutations in the C-terminal regions of FlhB, YscU, and homologs can restore the wild-type phenotype in T3S4 mutants of *S.* Typhimurium, *Y. pseudotuberculosis*, EPEC, and *X. campestris* pv. vesicatoria (148, 298, 330, 602, 626) (Table 7). It is assumed that the introduction of point mutations into the C-terminal domains of YscU/FlhB family members leads to a conformational change that is permissive for the substrate specificity switch.

The C-terminal domains of YscU/FlhB family members are cleaved autoproteolytically between the asparagine and proline residues of a conserved NPTH motif (letters refer to amino acids) (38, 130, 177, 306, 334, 509, 597, 626). The C-terminal cleavage products probably remain associated with the membrane-associated portions of the proteins. Since the motility of a flagellar $flhB_{CC}$ mutant, which lacks the C-terminal cleavage product of FlhB, can be restored partially upon ectopic expression of $flhB_{CC}$, it was suggested that the cleavage product can be provided in *trans* and that the cleavage event *per se* is not crucial for protein function (381, 583). Similar findings were observed for an *X. campestris* pv. vesicatoria $hrcU_{CC}$ mutant (330). Crystal structure analyses of several YscU/FlhB family members revealed that the cleavage leads to an altered orientation of the PTH loop, while the rest of the C-terminal protein domain remains structurally unchanged (38, 130, 148, 177, 334, 597, 602, 626). Mutant derivatives of YscU/FlhB family members which carry point mutations in the conserved N, P, or T residue of the NPTH motif are no longer efficiently cleaved but still promote the secretion of early substrates and needle formation (Table 7). Furthermore, the lack of YscU/FlhB cleavage does not significantly compromise the secretion of T3S4 proteins (509, 626) (Table 7). In contrast, secretion of intermediate and late substrates is often suppressed in YscU/FlhB cleavage mutants, suggesting that the cleavage of YscU/FlhB family members, and thus the reorientation of the PTH loop, is required for the substrate specificity switch (186, 330, 507, 509, 548, 626) (Table 7). In EPEC and *X. campestris* pv. vesicatoria, the presence of noncleavable EscU and HrcU derivatives, respectively, leads to a significantly reduced secretion of effector proteins (330, 548) (Table 7). Since the IM association of the T3S chaperone CesT is reduced in EscU cleavage mutants, it was proposed that the cleavage is required for substrate docking (548). In agreement with this hypothesis, mutations in the NPTH motif of HrcU interfere with the interaction of HrcU with the early T3S substrate HrpB2 (330).

In contrast to EPEC and *X. campestris* pv. vesicatoria, *Yersinia* sp. mutants that are deficient in YscU cleavage still secrete effector proteins as well as hybrid proteins consisting of the N-terminal T3S signal of the effector protein YopE and the tip protein LcrV (38, 509) (Table 7). Secretion of the wild-type LcrV protein, in contrast, was severely reduced in the absence of YscU cleavage (509). YscU cleavage might therefore be required to activate the secretion of translocon proteins (intermediate substrates) but not effector proteins (late substrates). Furthermore, these data indicate that the classification of T3S substrates as early, intermediate, or late substrates depends on the N-terminal T3S signal, which might determine the time point of secretion. In agreement with this hypothesis, the results of domain swapping experiments with substrates of the flagellar T3S system revealed that hybrid proteins that contain the N-terminal regions of early substrates are also secreted as early substrates (517). Similarly, the secretion behavior of translocon and effector proteins in *sepD* or *sepL* mutants (see below) of EPEC was determined by the N-terminal T3S signal (398). To date, different models for the substrate specificity switch

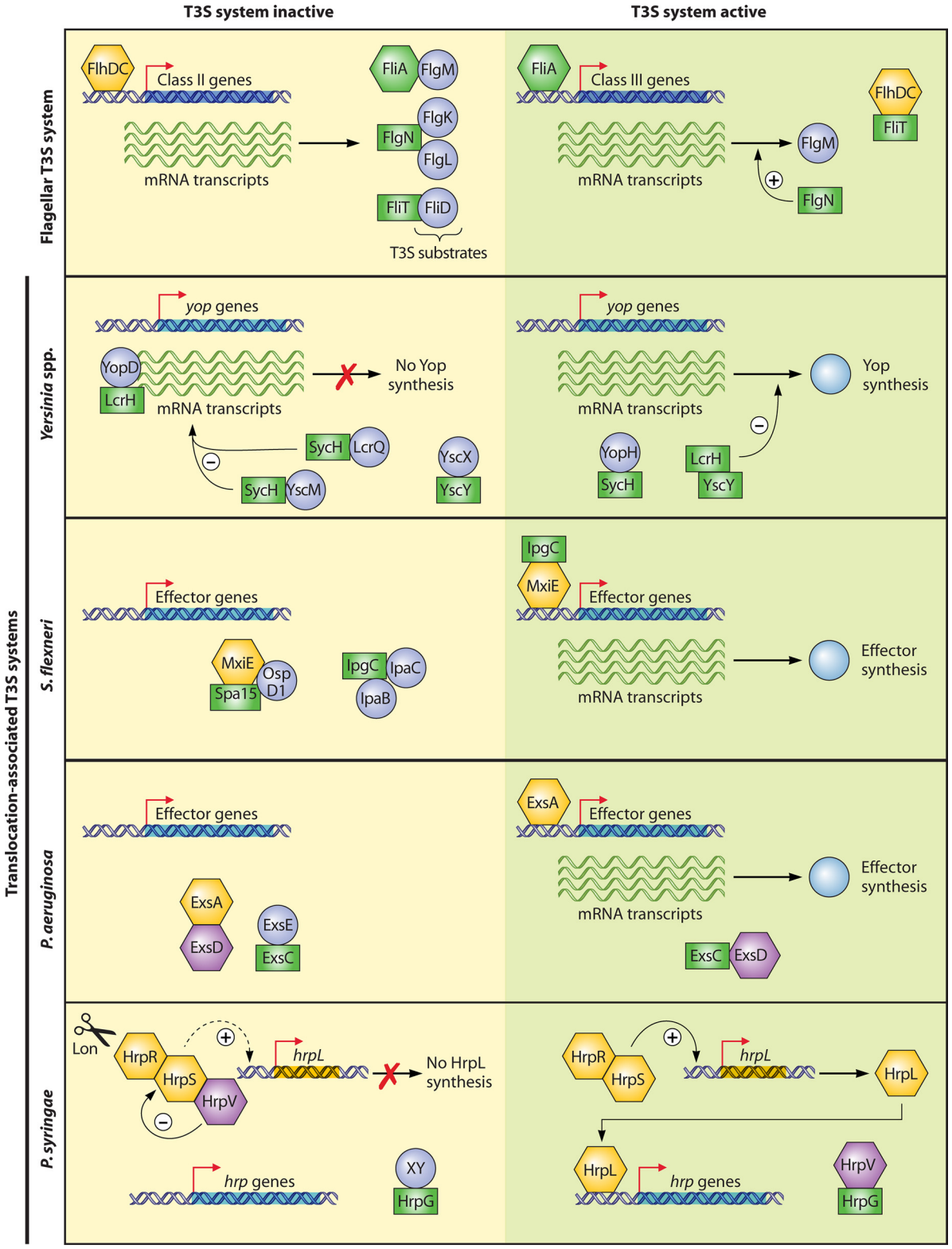


FIG 7 Schematic representation of the regulatory mechanisms that control T3S gene expression in flagellar and translocation-associated T3S systems. The expression of effector genes or genes encoding substrates and components of the flagellar T3S system is controlled by transcriptional regulators and regulatory chaperones and depends on the activity of the T3S system. Flagellar gene expression is regulated by the transcriptional activators FlhDC, which are encoded by class I genes and activate the expression of class II genes. Class II gene products include the anti-σ^{28} factor FlgM, the hook-filament junction proteins FlgK and FlgL, and the filament cap protein FliD, which bind to the corresponding chaperones FliA, FlgN, and FliT, respectively. The secretion of FlgM leads to the release of the σ^{28} factor FliA, which activates the expression of class III genes. The liberated T3S chaperone FliT binds to FlhC and thus inhibits the expression of class II genes, whereas FlgN positively regulates the translation of *flgM* class III mRNA. In *Yersinia* spp., translation of *yop* mRNAs is suppressed by a YopD-LcrH

TABLE 6 Characteristics and functions of T3S4 proteins

T3S4 protein (organism)	Protein length (aa)	Needle/pilus/hook length (nm [unless stated otherwise])	T3S of T3S4 protein	Interaction with $YscU_C$/$FlhB_C$ or homologs	Mutant phenotype	References
Translocation-associated T3S systems of animal-pathogenic bacteria						
YscP (*Yersinia* spp.)	515	55–60	Yes	Not shown	Reduced secretion of effector proteins; increased amounts of surface-localized YscF; increased secretion of the predicted inner rod protein YscI; lack of needle length control	148, 257, 518, 605
InvJ (*Salmonella* spp.)	336	≈80	Yes	Not shown	Lack of inner rod structures in isolated injectisomes; wild-type secretion of PrgI (needle protein) and PrgJ (inner rod protein); needles are loosely attached to the base; lack of needle length control	98, 204, 293, 347, 528
Spa32 (*S. flexneri*)	292	≈45	Yes	Yes	Reduced secretion of IpaB, IpaC, and IpaD; lack of needle length control	50, 341
Translocation-associated T3S systems of plant-pathogenic bacteria						
HpaC (*X. campestris* pv. vesicatoria)	212	≈1–2 μm	No	Yes	Reduced secretion of translocon and effector proteins; increased secretion of HrpB2	332, 496
Flagellar T3S systems of animal-pathogenic bacteria						
FliK (*Salmonella* spp.)	405	≈55	Yes	Yes	Elongated hook structures; no filaments	374, 386, 392, 433, 536

in translocation-associated and flagellar T3S systems have been proposed, and these are discussed below.

T3S Substrate Specificity Switching in Translocation-Associated T3S Systems

The molecular ruler model proposed for the T3S4 protein YscP from *Yersinia* spp. Given the finding that T3S4 proteins from animal-pathogenic bacteria determine needle length and are secreted by the T3S system, they were proposed to act as molecular rulers that measure needle length. This so-called molecular ruler model was based on the finding that deletions and insertions in the T3S4 protein YscP result in shorter and longer needle structures, respectively (257). The simultaneous production of a short and a long version of YscP led to two different needle populations with corresponding lengths but not intermediate sizes. It was therefore assumed that the length of each needle is controlled by only one, not several, YscP molecules (single ruler model) (579). The molecular ruler model assumes that the C-terminal region of T3S4 proteins remains attached to the base of the secretion apparatus while the N-terminal portion travels the inner channel of the needle (Fig. 8A). Since an interaction between YscP and the needle protein YscF or the needle tip protein LcrV has not yet been demonstrated, the identity of YscP docking sites at the tip of the growing needle still remains to be identified. Once the ruler is stretched, the C-terminal T3S4 domain signals the switch in T3S substrate specificity, probably via interaction with the C-terminal domain of YscU, and thus activates the secretion of translocon proteins.

complex when the T3S system is inactive. LcrQ-SycH and YscM-SycH complexes might act as additional repressors. Activation of Yop secretion leads to relief of the YopD-LcrH-mediated repression of *yop* mRNA translation and the liberation of SycH and LcrH upon secretion of YopD. The SycH chaperone presumably promotes YopH secretion after its release from the secreted regulator YscM. LcrH might suppress *yop* gene expression when bound to the T3S chaperone YscY. Effector gene expression in *S. flexneri* depends on the transcriptional activator MxiE and its coactivator, IpgC. Upon activation of T3S, MxiE and IpgC are released from their secreted interaction partners, i.e., Spa15, OpsD1, IpaC, and IpaD, and can activate effector gene expression. In *P. aeruginosa*, effector gene expression is controlled by the activator ExsA, which interacts with the antiactivator ExsD when the T3S system is inactive. The T3S chaperone ExsC, which binds to the T3S substrate ExsE, acts as an antiantiactivator when released from ExsE after activation of the T3S system. The interaction of ExsC with ExsD leads to the liberation of ExsA, which subsequently activates effector gene expression. In the plant-pathogenic bacterium *P. syringae*, expression of *hrp* (hypersensitive response and pathogenicity) genes that encode the T3S system is induced by HrpR, HrpS, and HrpL. HrpR and HrpS interact with each other and activate the expression of the alternative sigma factor HrpL, which binds to the promoters of *hrp* genes. HrpR-, HrpS-, and HrpL-dependent activation of *hrp* gene expression is counteracted by the negative regulator HrpV, which interacts with HrpS, and by the Lon protease, which degrades HrpR. Under T3S-inducing conditions, HrpV interacts with the chaperone-like protein HrpG, which interferes with the negative regulatory activity of HrpV and might also bind to a secreted but so far unknown interaction partner (XY). T3S chaperones are represented in green, and T3S substrates are represented by circles. Transcriptional regulators are depicted in yellow.

TABLE 7 Effects of point mutations or deletions in YscU/FlhB family members

YscU/FlhB homolog (organism)	Mutation (expression in *trans* or in *cis*)	Characteristics of mutated YscU/FlhB homologs		Reference(s)
		Cleavage[a]	Effect on T3S	
YscU (*Yersinia* spp.)	Mutations in the NPTH motif			
	N263A (in *cis*)	Reduced/alternative cleavage	Secretion of Yops and LcrQ in the presence of Ca^{2+}; reduced secretion of Yops and LcrV in the absence of Ca^{2+}; increased amounts of surface-exposed YscF; secretion of YscP is not significantly altered	38, 509
	N263A (in *trans*)	Alternative/partial cleavage	Reduced secretion of LcrV and translocon and effector proteins; longer needles	457, 509
	P264A (in *cis*)	Reduced/alternative cleavage	Secretion of Yops in the presence and absence of Ca^{2+}; reduced secretion of effector and translocon proteins in the absence of Ca^{2+}; reduced secretion and expression of LcrV; increased amounts of surface-exposed YscF	38, 509
	P264A (in *trans*)	No/alternative cleavage	Reduced secretion of effector and translocon proteins; longer needles	306, 509
	T265A (in *cis*)	Wild type	Wild-type phenotype	38
	T265A (in *trans*)	Wild type	Wild-type phenotype	509
	H266A (in *trans*)	Reduced cleavage	Secretion of LcrV; wild-type needle length	597
	ΔNPTH (in *cis*/in *trans*)	No cleavage	No secretion of effectors; low level of effector gene expression; no surface-exposed YscF	38, 306
	Extragenic mutations that suppress the *yscP* mutant phenotype			
	A268F (in *trans*)	NA	Restores effector protein secretion in a *yscU* mutant, but not secretion of the needle protein YscF	148
	Y287G (in *trans*)	NA	Partially restores effector protein secretion in a *yscU* mutant; leads to reduced amounts of surface-localized YscF in a *yscP* mutant; does not restore wild-type levels of YscI secretion in a *yscP* mutant	148, 605
	V292T (in *trans*)	NA	Restores effector protein secretion in a *yscU* mutant	148
	Y317D (in *trans*)	NA	Does not restore effector protein secretion in a *yscU* mutant but does so in a *yscP yscU* double mutant; leads to reduced amounts of surface-localized YscF in a *yscP* mutant; leads to reduced YscI secretion in a *yscP* mutant	148, 605
EscU (EPEC)	Mutations in the NPTH motif			
	N262A (in *trans*)	No cleavage	Reduced/no secretion of effector and translocon proteins	626
	N262D (in *trans*)	Reduced cleavage	Reduced/no secretion of effector and translocon proteins	626
	P263A (in *trans*)	No cleavage	Reduced/no secretion of effector and translocon proteins	626
	H265A (in *trans*)	Cleavage	Reduced/no secretion of effector and translocon proteins	626
SpaS (*Salmonella* spp.)	Mutations in the NPTH motif			
	N258A (in *cis*)	NA	Reduced secretion of needle, translocon, and effector proteins; secretion of the T3S4 protein InvJ is not significantly altered	626
	P259A (in *cis*)	NA	Reduced secretion of needle, translocon, and effector proteins; secretion of the T3S4 protein InvJ is not significantly altered	626

(Continued on following page)

TABLE 7 (Continued)

YscU/FlhB homolog (organism)	Mutation (expression in *trans* or in *cis*)	Characteristics of mutated YscU/FlhB homologs		Reference(s)
		Cleavage[a]	Effect on T3S	
HrcU (*X. campestris* pv. vesicatoria)	Mutations in the NPTH motif			
	N264A (in *trans*)	No cleavage	No secretion of translocon and effector proteins; wild-type secretion of the early T3S substrate HrpB2; interacts with HrpB2; reduced interaction with the T3S4 protein HpaC	330
	P265A (in *trans*)	Reduced cleavage	Reduced secretion of translocon and effector proteins; wild-type secretion of the early T3S substrate HrpB2; interacts with HrpB2; reduced interaction with the T3S4 protein HpaC	330
	P265G (in *trans*)	No cleavage	No secretion of translocon proteins, effectors, and the early T3S substrate HrpB2; reduced interaction with HrpB2 and the T3S4 protein HpaC	330
	P265G (in *cis*)	No cleavage	No secretion of translocon and effector proteins and the early T3S substrate HrpB2	330
	T266A (in *trans*)	Cleavage	Wild-type secretion of translocon and effector proteins and the early T3S substrate HrpB2	330
	H267A (in *trans*)	Cleavage	Reduced secretion of translocon and effector proteins; wild-type secretion of the early T3S substrate HrpB2	330
	Extragenic suppressor mutation that suppresses the *hpaC* mutant phenotype			
	Y318D (in *cis*)	Reduced cleavage	Wild-type secretion of translocon and effector proteins; oversecretion of the early T3S substrate HrpB2 in the *hpaC* deletion mutant is not restored; reduced interaction with the T3S4 protein HpaC and with HrpB2	330
FlhB (*Salmonella* spp.)	Mutations in the NPTH motif			
	N269A (in *trans*)	Alternative cleavage	Polyhooks	186
	P279A (in *trans*)	Alternative cleavage	Polyhooks	186
	Extragenic suppressor mutations that suppress the *fliK* mutant phenotype			
	A298V (in *trans*)	Reduced cleavage	Partially restores motility of *fliK* mutant (to less than 25% of wild-type motility)	381, 602
	G293R (in *trans*)	Reduced cleavage	Partially restores motility of *fliK* mutant (to less than 25% of wild-type motility)	381, 602
	G293V (in *trans*)	Reduced cleavage	Partially restores motility of *fliK* mutant (to less than 25% of wild-type motility)	381, 602
	348 frameshift (in *trans*)	Reduced cleavage	Partially restores motility of *fliK* mutant (to less than 25% of wild-type motility)	381, 602
	358 frameshift (in *trans*)	Reduced cleavage	Partially restores motility of *fliK* mutant (to less than 25% of wild-type motility)	381, 602
	W353stop (in *trans*)	Reduced cleavage	Partially restores motility of *fliK* mutant (to less than 25% of wild-type motility)	381, 602
FlhB (*H. pylori*)	Mutations in the NPTH motif			
	N265A (in *cis*)	No cleavage	No secretion of filament proteins	507
	P266G (in *cis*)	No cleavage	No secretion of filament proteins	507

[a] NA, not analyzed.

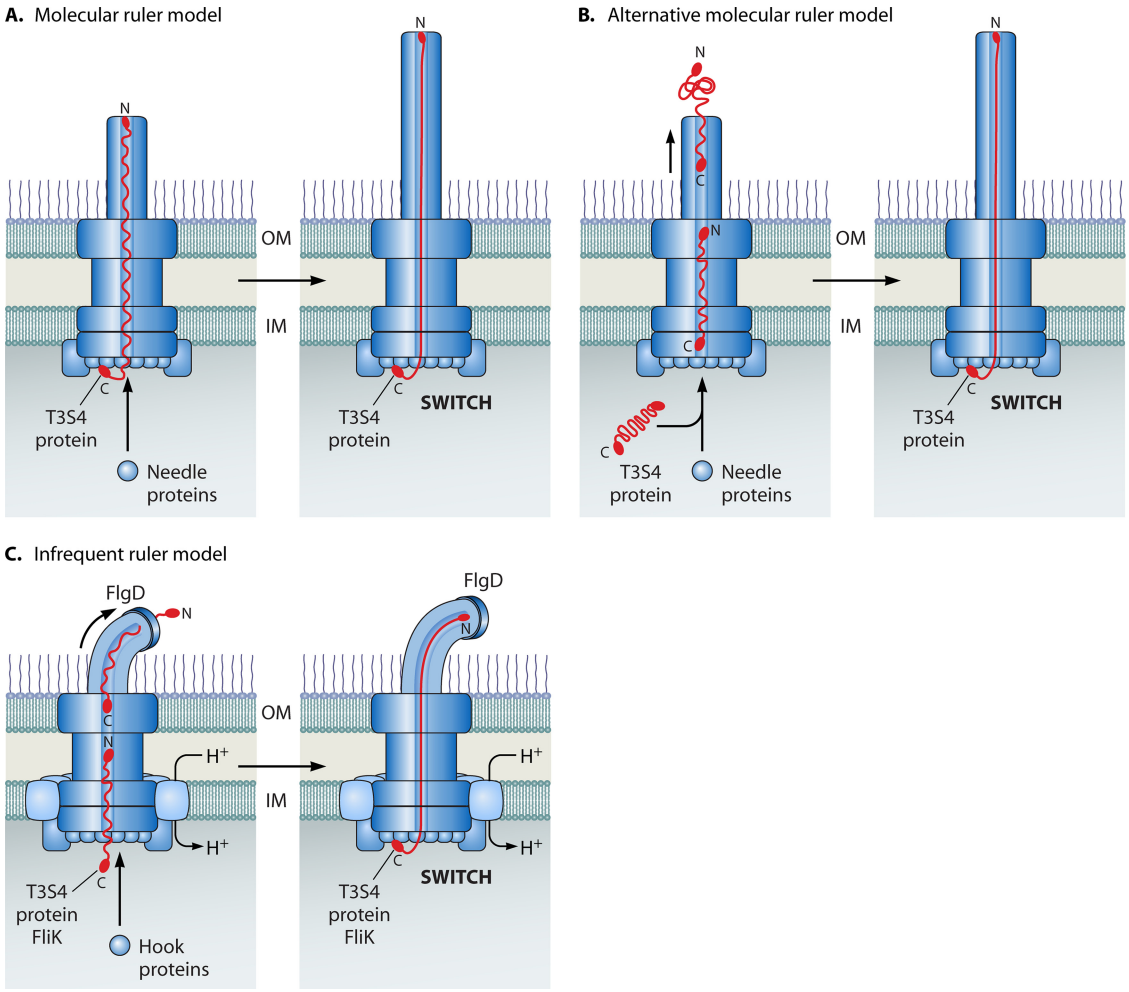


FIG 8 Proposed modes of action of T3S4 proteins from animal-pathogenic bacteria. (A) Molecular ruler model. According to the molecular ruler model, the N terminus of the T3S4 protein is attached to the tip of the growing needle. Once the T3S4 protein is stretched, the C-terminal region signals the substrate specificity switch via interaction with the C-terminal domain of a member of the YscU/FlhB family. N, N-terminal region; C, C-terminal region. (B) Alternative molecular ruler model. This model predicts that T3S4 proteins are constantly secreted during needle assembly and thereby measure needle length. The interaction between the C-terminal region of the T3S4 protein and the C-terminal domains of members of the YscU/FlhB family leads to a switch in the substrate specificity and occurs only when the needle has reached a certain length. (C) Infrequent ruler model proposed for flagellar T3S systems. During hook assembly, the T3S4 protein FliK is intermittently secreted and temporarily interacts with hook components such as the hook-capping protein FlgD. However, the rapid secretion of FliK does not allow a productive interaction of the C-terminal domain of FliK with the C-terminal domain of FlhB. After the hook has reached its physiological length of approximately 55 nm, the N-terminal region of FliK interacts more frequently with hook subunits and the reduced secretion rate of FliK allows an interaction of the C-terminal region of FliK with the C-terminal domain of FlhB, and thus the induction of the substrate specificity switch.

Notably, the molecular ruler model predicts that YscP and the needle protein YscF are secreted at the same time. Since the average width of YscP in an extended alpha-helical conformation was calculated to be 1 to 1.3 nm and the inner channel of the secretion apparatus has a diameter of approximately 2 to 3 nm, it might be just sufficiently wide enough to allow the passage of two partially (YscP) or completely (YscF?) unfolded proteins (578).

Possible contribution of the predicted inner rod to the substrate specificity switch. An alternative mechanism to the molecular ruler model was proposed and considers a possible contribution of the predicted inner rod structure to the substrate specificity switch in *Yersinia* spp. It was shown that the absence of the T3S4 protein YscP leads to oversecretion of the putative inner rod protein YscI (605). Since the introduction of point mutations into YscU not only suppresses the *yscP* mutant phenotype but also restores wild-type levels of YscI secretion, it was suggested that the substrate specificity switch is linked to the control of YscI secretion (605). YscP might therefore control the assembly of the predicted inner rod, which could be required for the T3S substrate specificity switch (605). The potential contribution of T3S4 proteins to inner rod formation is supported by the finding that the absence of the T3S4 protein InvJ in *Salmonella* spp. leads to structural differences in the base of the T3S system that are presumably caused by a disturbance of the inner rod formation (347). Furthermore, the amounts of the inner rod protein PrgJ associated with the needle complex are significantly reduced in *invJ* mutants (528). Although the possible contribution of the predicted inner rod assembly to the T3S substrate specificity switch is at odds with the molecular ruler model, it cannot be excluded that a

combination of both mechanisms is involved in the control of T3S.

Substrate specificity switching by the T3S4 protein Spa32 from *S. flexneri*. The different sizes of T3S4 proteins from animal-pathogenic bacteria do not always correlate with the observed differences in needle length (Table 6). The T3S4 protein Spa32 from *S. flexneri*, for instance, is 292 amino acids long, compared with 515 amino acids for YscP from *Yersinia* spp. However, T3S needles from *S. flexneri* are only approximately 20% shorter than the needles from *Yersinia* spp. (Table 6). Furthermore, deletions within Spa32 do not lead to a reduction in needle length (50). Notably, however, Spa32 is functionally interchangeable with YscP from *Yersinia* spp. and InvJ (336 amino acids) from *Salmonella* spp. (50). In contrast, a Spa32-YscP hybrid protein containing the central ruler domain of YscP flanked by the N- and C-terminal regions of Spa32 led to a 2-fold increase in needle length in *S. flexneri* compared with that observed with the native YscP or Spa32 protein (50). It is still unclear why the ruler region of YscP leads to longer needles in the context of a Spa32-YscP hybrid but not in the wild-type protein. Since it was shown that not only the length but also the helical structure of YscP might contribute to needle length control (578), it remains to be investigated whether differences in the secondary structures of YscP and Spa32-YscP hybrid proteins could account for the differences in needle length.

The analysis of Spa32 truncation derivatives revealed that the N- and C-terminal protein regions are required for protein function. The C-terminal protein region harbors the binding site for the YscU/FlhB homolog Spa40, while the N-terminal protein portion travels the inner channel of the needle (50). It was therefore proposed that Spa32 is constantly secreted during needle assembly and signals the switch via the interaction with the C-terminal domain of Spa40 once the needle has reached its final length (50) (Fig. 8B).

Substrate specificity switching during T3S in the plant-pathogenic bacterium *X. campestris* pv. vesicatoria. While T3S4 proteins have been studied intensively in animal-pathogenic bacteria, less is known about the molecular mechanisms underlying T3S substrate specificity switching in plant pathogens. Functional studies of T3S4 proteins have so far been performed only with *X. campestris* pv. vesicatoria (332). In contrast to T3S4 proteins from animal pathogens, HpaC from *X. campestris* pv. vesicatoria is a cytoplasmic protein and therefore does not act as a secreted molecular ruler (73). So far, there is no experimental evidence for length control of the extracellular T3S pilus for plant-pathogenic bacteria. Since the T3S pilus is significantly longer (up to 2 μm) than the needle from animal-pathogenic bacteria, it probably cannot be bridged by a single molecular ruler molecule, suggesting that T3S substrate specificity switching in plant-pathogenic bacteria is not linked to length control of the pilus.

The T3S4 protein HpaC switches the substrate specificity of the T3S system from the early T3S substrate HrpB2 to translocon and effector proteins (332, 468). HrpB2 is essential for pilus formation and interacts with HpaC and the C-terminal domain of the YscU/FlhB homolog HrcU ($HrcU_C$), which also provides a binding site for HpaC (330, 332, 496, 591). Experimental evidence suggests that the $HrcU_C$-HrpB2 interaction is required for the efficient secretion of HrpB2 prior to the substrate specificity switch, which is in agreement with the predicted role of $HrcU_C$ as a substrate acceptor site (330). Since the NPTH motif of HrcU appears to be essential for the interaction of $HrcU_C$ with HpaC and HrpB2, both proteins might compete for the same binding site in $HrcU_C$ (330, 332, 496). It is therefore possible that HpaC prevents the efficient binding of HrpB2 to $HrcU_C$ and thus promotes the recognition of effector and translocon proteins by $HrcU_C$. However, it is still unknown whether $HrcU_C$ also acts as a substrate acceptor site for late substrates, because an interaction between $HrcU_C$ and effector proteins has not yet been observed (332).

Interestingly, the lack of substrate specificity switching in the absence of HpaC can be restored upon introduction of a point mutation into $HrcU_C$ (330). As mentioned above, point mutations in the C-terminal domains of YscU/FlhB family members could mimic a conformational change in these domains that is permissive for the secretion of late substrates. Notably, however, the increased secretion of the early substrate HrpB2 in the *hpaC* deletion mutant was unaltered in the presence of the suppressor mutation in HrcU, suggesting that secretion of early and late substrates from *X. campestris* pv. vesicatoria is controlled by independent mechanisms (330). Taken together, these studies reveal differences and similarities in the control mechanisms underlying T3S in plant- and animal-pathogenic bacteria. One major difference is the apparent lack of a secreted molecular ruler in *X. campestris* pv. vesicatoria. Furthermore, the finding that secretion of early and late T3S substrates from *X. campestris* pv. vesicatoria is controlled by different mechanisms that can be uncoupled has not been reported for animal-pathogenic bacteria.

T3S Substrate Specificity Switching in Flagellar T3S Systems

The flagellar T3S4 protein FliK presumably acts as an infrequent ruler. The length of the flagellar hook varies from 35 to 75 nm, with a peak at 55 nm, and is controlled by the T3S4 protein FliK, which is secreted during hook assembly (222, 374). Mutation of *fliK* results in elongated rod and hook structures and in a loss of filament formation (222, 433) (Table 6). Since insertions and deletions outside the C-terminal T3S4 domain of FliK lead to increased and reduced hook lengths, respectively, FliK most likely acts as a molecular ruler, as proposed for YscP from *Yersinia* spp. (502). Interestingly, experimental evidence suggests that FliK is involved not only in hook length control but also in length control of the inner rod of the flagellar T3S system (538).

Similar to the case in translocation-associated T3S systems, the substrate specificity switch in flagellar T3S systems is induced when the C-terminal T3S4 domain of FliK ($FliK_C$) interacts with the C-terminal domain of FlhB. As mentioned above, this interaction is probably favored at a hook length of approximately 55 nm (159, 381, 383, 392). Prior to hook completion, binding of $FliK_C$ to $FlhB_C$ is suppressed by an additional regulatory protein, termed RflH/Flk or Fluke (to clearly distinguish it from FliK), which prevents the premature secretion of filament components and is anchored in the IM (7, 265, 296, 385).

Interaction studies revealed that FliK binds to the hook-capping protein FlgD, which might provide the docking site for FliK within the growing hook structure (385, 391). It is still unclear whether the inner diameter of the hook, which is smaller than 2 nm, allows the simultaneous passage of FliK and the hook protein FlgE (498). It was therefore proposed that FliK acts as a more flexible ruler molecule that is constantly secreted through the growing hook structure and switches the substrate specificity when the hook has reached its final length (159) (Fig. 8C). This so-called infrequent ruler model, which predicts temporal mea-

surements of the hook length by intermittently secreted FliK molecules, was recently supported by experiments in *S. enterica* in which *fliK* expression and hook polymerization were uncoupled. It was shown that the substrate specificity switch occurred immediately when *fliK* expression was induced in a strain with elongated hook structures (162). Furthermore, the simultaneous production of a short and a long FliK derivative resulted in short hooks corresponding to the short FliK molecule, in agreement with the infrequent ruler model (162). Thus, since the substrate specificity switch depends on the interaction between the C-terminal regions of FliK and FlhB, the first FliK molecule that travels a hook with an appropriate length will signal the switch to the secretion of filament proteins. In contrast, as mentioned above, a similar experiment performed with *Yersinia* spp. led to two populations, with short and long needles, suggesting that needle length in *Yersinia* spp. is controlled by a single static ruler molecule (579).

Interestingly, it was previously reported that overexpression of a secretion-deficient FliK derivative lacking the N-terminal 99 amino acids still allows secretion of the filament protein FliC but results in elongated hooks and severely reduced bacterial motility (221, 385). It was therefore speculated that increased amounts of an N-terminally truncated FliK derivative enable $FliK_C$ to interact with $FlhB_C$ and thus to signal the switch even in the absence of FliK secretion. In agreement with this model, substrate specificity switching by $FliK_{\Delta 1-99}$ was increased in the absence of Fluke (385). Notably, deletions in the central part (amino acids 208 to 278) of FliK, outside the T3S4 domain (amino acids 265 to 405), did not abolish filament formation and hook length control (502). Since these FliK derivatives were initially not detected in the culture supernatant, FliK was proposed to act as an internal ruler (502). However, secretion of FliK derivatives with deletions in the central protein region was shown in a later study by the use of a more sensitive FliK-specific antibody (159).

The measuring cup model. While the molecular ruler or tape measure model is now a widely accepted working hypothesis for FliK function, hook length control was initially considered to be controlled by the capacity of the C ring to be filled with a defined amount of the hook protein FlgE (measuring cup model). This theory was based on the observation that the lack of the C-ring component FliG, FliM, or FliN led to shorter hooks (342). According to the measuring cup model, emptying the C ring would allow access of FliK to $FlhB_C$ and thus would allow the switch to occur. However, the C ring can be filled with 50 FlgE hook subunits at most, while at least 120 subunits are required to reach the average hook length (342, 476). This implies that the C ring would have to be emptied several times before the substrate specificity switch occurs. Furthermore, the measuring cup model does not explain the finding that mutations in the hook-capping protein FlgD abolish the switch in T3S substrate specificity. *flgD* mutants are nonmotile and do not assemble the hook, but they secrete the hook protein FlgE (380, 416). According to the measuring cup model, the substrate specificity switch should occur when FlgE is secreted, even in the absence of FlgD. Since this is not the case, the predicted measuring device provided by the C ring is not sufficient to account for the switch in substrate specificity that activates filament formation (and thus motility) after hook assembly. In agreeement with this is the observation that hook length control and filament formation are not abolished in the absence of the C ring (159).

The molecular clock model. Besides the molecular ruler and measuring cup models, yet another mechanism involving a molecular timing device was proposed to explain substrate specificity switching in flagellar T3S systems. According to this so-called molecular clock model, initiation of hook assembly activates a countdown which signals the switch in substrate specificity after the hook has reached a length of approximately 55 nm (391). This model was based on the observation that the length of polyhooks in *fliK* mutants still peaks at 55 nm, suggesting that hook length control does not depend solely on the molecular ruler function of FliK (285, 391). Since mutant derivatives of the hook protein FlgE that had a defect in polymerization resulted in shorter hooks, it was suggested that the hook polymerization rate determines the time point of the substrate specificity switch (391).

One potential mechanism that was proposed to serve as a molecular clock was the proteolytic cleavage of FlhB, which has a half-life of approximately 7 min (177, 391). Notably, however, the cleavage event itself is required but presumably not essential for the substrate specificity switch, because coproduction of both FlhB cleavage products can partially restore the motility of *flhB* mutants, as shown for *Salmonella* spp. and *Helicobacter pylori* (381, 583) (see above). Furthermore, the finding that polymerization-deficient FlgE mutant derivatives result in shorter hooks can be explained not only by a predicted molecular clock mechanism but also by the enhanced FliK secretion that was observed in these strains (391). According to the infrequent molecular ruler model, a high secretion rate of FliK increases the probability of an interaction between FliK and $FlhB_C$. Since this interaction induces the substrate specificity switch, the switch might occur earlier in strains that oversecrete FliK, thus leading to shorter hooks (162). In agreement with this model, overexpression of FliK was previously shown to result in shorter hooks (399). In conclusion, the experimental data published to date on mechanisms underlying flagellar hook length control support the infrequent molecular ruler model rather than the molecular cup or molecular clock model.

A SECOND SWITCH ACTIVATES EFFECTOR PROTEIN SECRETION

In translocation-associated T3S systems, secretion is controlled not only by T3S4 proteins and transcriptional regulators but also by posttranscriptional mechanisms that might impose a hierarchy on the secretion of intermediate and late substrates. Since the insertion of the translocon is a prerequisite for effector protein translocation, there is probably a second switch in the T3S substrate specificity that activates effector protein secretion after translocon assembly. This hypothesis is supported by the finding that the secretion of translocon proteins requires a different trigger (e.g., serum albumin in *Y. enterocolitica* [313] and bile salts in *S. flexneri* [418]) from that for the secretion of effector proteins (e.g., 37°C or a low calcium level in *Yersinia* spp. [368, 526] and Congo red in *S. flexneri* [432]). Although far from being understood, several control mechanisms that underlie the secretion of late substrates have been described for translocation-associated T3S systems from animal-pathogenic bacteria. In several cases, the transit of effector proteins through the secretion channel is physically blocked by a gatekeeper protein until the formation of the translocon is completed. The trigger that activates effector protein secretion could be the contact with the host cell, which is sensed by the needle or the needle tip complex. Transduction of the signal to

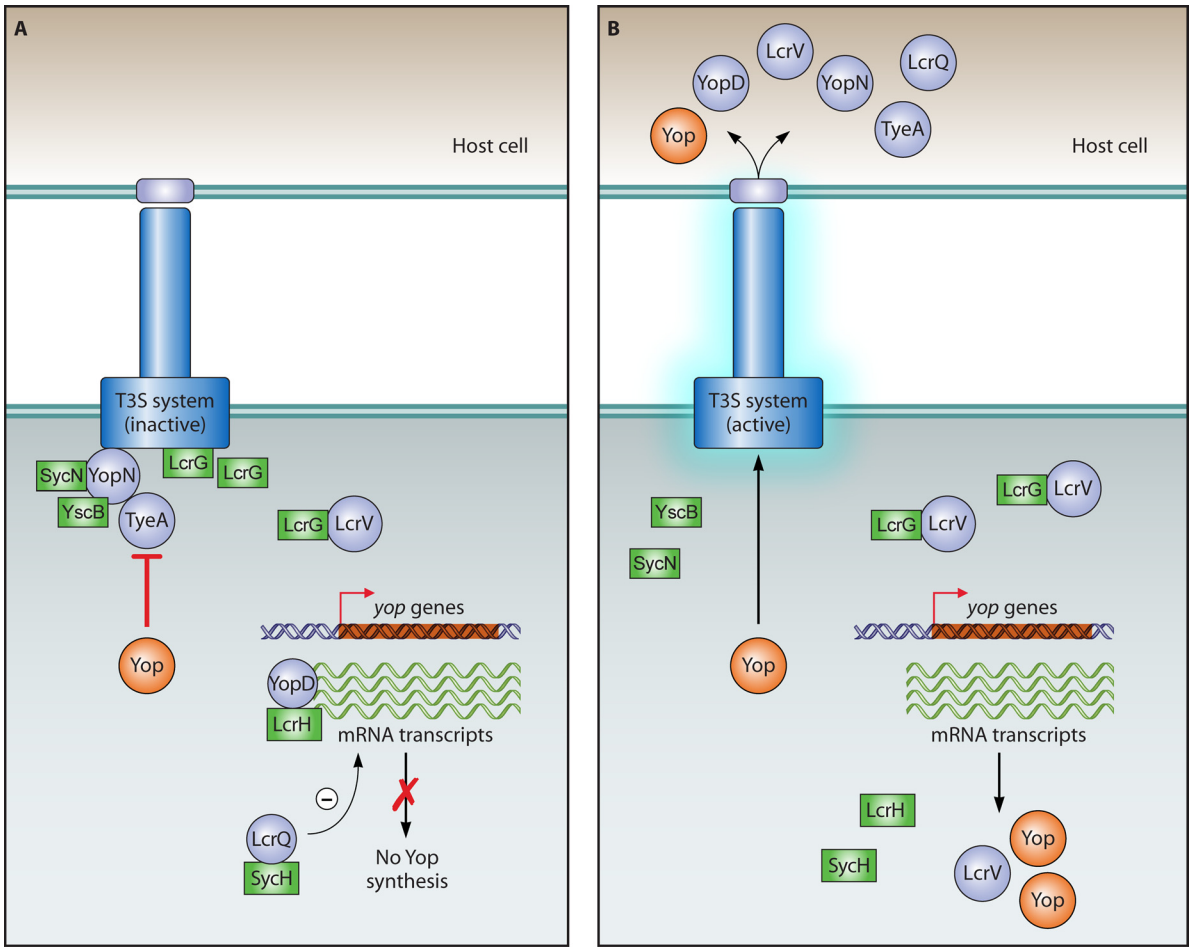


FIG 9 Model of the YopN-mediated control of effector protein secretion in *Yersinia* spp. (A) A complex of YopN, TyeA, and the YopN-specific chaperones SycN and YscB blocks the transit of T3S substrates (represented by circles) through the secretion apparatus. The T3S chaperone LcrG, which interacts with the tip protein LcrV, acts as an additional negative regulator of Yop secretion. YopD-LcrH and LcrQ-SycH complexes suppress *yop* gene expression when the T3S is inactive (see Fig. 7). (B) Activation of Yop secretion. The activation of the T3S system leads to the secretion of YopD, YopN, TyeA, LcrQ, and LcrV. This relieves the negative effect of YopN and TyeA on Yop secretion as well as of YopD and LcrQ on *yop* gene expression and thus leads to increased synthesis of Yops, including LcrV. Increased numbers of LcrV proteins bind to LcrG and presumably suppress the negative influence of LcrG on T3S.

the base of the secretion apparatus by the needle subunits might relieve the inhibitory effect of the gatekeeper protein and activate effector protein secretion. A brief summary of known gatekeeper and control proteins that are involved in the regulation of effector protein secretion is given below.

Control of Effector Protein Secretion in *Yersinia* spp. and *P. aeruginosa* by YopN Family Members

The secretion of Yops in *Yersinia* spp. is controlled by at least six different proteins, including the negative regulator YopN, its T3S chaperones SycN and YscB, the YopN-interacting protein TyeA, and the additional negative regulator LcrG, which binds to the tip complex protein LcrV (Table 5; Fig. 9) (212). YopN interacts with TyeA and a SycN-YscB complex in the bacterial cytosol and blocks translocon and effector protein secretion, presumably by preventing the transit of these proteins through the inner channel of the T3S system (89, 91, 126, 176) (Fig. 9A). Deletion of *tyeA*, *yopN*, *sycN*, or *yscB* therefore results in constitutive Yop secretion (212). The cytosolic TyeA protein prevents the secretion of YopN and thus the activation of effector protein secretion (89, 532). Upon host cell contact, however, secretion and translocation of YopN are triggered by a signal that might be transmitted via the needle to the secretion apparatus and thus abolishes the inhibitory effect of YopN on T3S (176, 552) (Fig. 9B).

A similar regulatory role was proposed for LcrG, the second negative regulator of Yop secretion in *Yersinia* spp. (407, 454). The regulatory activity of LcrG is counteracted by the tip complex protein LcrV, which interacts with LcrG in the bacterial cytoplasm (133, 351, 406, 407). According to the LcrG titration model, the induction of T3S leads to the activation of *lcrV* expression (see above) and thus to increased levels of LcrV that bind to LcrG and counteract its inhibitory effect on Yop secretion (351) (Fig. 9B). Although LcrG can block Yop secretion in the absence of YopN, there might be a molecular cross talk between both regulators. It was proposed that the negative influence of YopN on the secretion of LcrV indirectly regulates the function of LcrG (212).

Control of effector protein secretion by a negative regulator that might serve as a specific "plug" of the secretion channel was also reported for *P. aeruginosa*. The YopN homolog PopN from *P.*

TABLE 8 Contributions of selected mutations in needle, translocon, and tip complex proteins to the control of T3S

Needle, translocon, or tip complex protein mutant(s)[a]	Organism	Effect on T3S	Reference(s)
Needle protein mutants			
$YscF_{D28A}$, $YscF_{D46A}$	*Yersinia* spp.	Constitutive secretion of effectors, translocon proteins, and YopN; larger amounts of extracellular Ca^{2+} are required to block T3S	552
$YscF_{K58R}$, $YscF_{A72V}$, $YscF_{N31A}$, $YscF_{T70A}$	*Yersinia* spp.	WT secretion of YopE and YscF; reduced translocation of YopE and reduced pore formation	124
$YscF_{D82G}$, $YscF_{N47S}$, $YscF_{N47S, N68S}$	*Yersinia* spp.	WT secretion of YopE and YscF; reduced translocation of YopE; WT pore formation	124
$MxiH_{P44A}$, $MxiH_{P51A}$	*S. flexneri*	Constitutive secretion of translocon and effector proteins; altered composition of IpaB, IpaC, and IpaD	268, 569
$MxiH_{D73A}$	*S. flexneri*	Constitutive secretion of translocon and effector proteins; T3S is not inducible by Congo red; needles lack IpaB, IpaC, and IpaD	268, 569
$MxiH_{K69A}$, $MxiH_{R83A}$	*S. flexneri*	WT secretion of translocon proteins, but no secretion of effector proteins upon induction with Congo red; no secretion of MxiC	268, 350
Translocon protein mutants			
Δ*yopD* mutant	*Yersinia* spp.	Deregulated Yop synthesis and secretion (secretion of LcrV and Yops in the presence of Ca^{2+}); no translocation of Yops	182, 465, 601
Δ*ipaB* mutant	*S. flexneri*	Unregulated secretion of translocon and effector proteins (no responsiveness to Congo red)	362, 461, 501, 569
Tip complex protein mutants			
Δ*lcrV* mutant	*Yersinia* spp.	Reduced secretion of effector proteins; unaltered secretion of YopB and YopD; no pore formation	133, 344
Δ*pcrV* mutant	*P. aeruginosa*	Increased secretion of effector proteins in the presence of Ca^{2+}; increased secretion of ExoS in the presence of eukaryotic cells; unaltered secretion of PopB and PopD; no assembly of the translocation pore	208, 310, 533
Δ*ipaD* mutant	*S. flexneri*	Unregulated secretion of Ipa and effector proteins (no responsiveness to Congo red)	362, 442, 461, 501, 569
Δ*sipD* mutant		Increased T3S, no translocation of effector proteins	260, 261

[a] Only selected point mutant derivatives are listed.

aeruginosa blocks the secretion of effector proteins before it is itself secreted and translocated (533, 611). Notably, however, in contrast to effector proteins, translocon proteins are still secreted under conditions that do not favor PopN secretion, suggesting that the negative influence of PopN on T3S is specific for effector proteins (95). In this context, it is interesting that the secretion of translocon proteins by the T3S system from *P. aeruginosa* appears to be constitutive (95).

The LcrG homolog PcrG from *P. aeruginosa* presumably blocks effector protein secretion from inside the cytoplasm by an unknown mechanism (533). PcrG interacts with PcrV, which is a homolog of LcrV and is secreted in a PcrG-dependent manner. Notably, however, the PcrG-mediated suppression of effector protein secretion is independent of its interaction with PcrV (310). This is different from the anticipated interplay between LcrV and LcrG (see above) and suggests that despite the presence of sequence-related control proteins, the regulatory mechanisms underlying effector protein export in *Yersinia* spp. and *P. aeruginosa* can vary significantly. Different regulatory mechanisms might also explain the finding that in contrast to the mutation of *lcrV*, which does not lead to deregulated secretion (32, 481), deletion of *pcrV* leads to constitutive effector protein secretion in *P. aeruginosa* (310, 407) (Tables 5 and 8). The PcrV-mediated control of effector protein secretion is probably linked to the role of PcrV as a tip complex protein, because PcrV has to be secreted to regulate T3S (310). However, it is unlikely that PcrV acts simply as an external plug, because translocon proteins are secreted prior to host cell contact, when effector protein secretion is probably off (95). It was therefore proposed that the assembly of the tip complex stabilizes an "off" conformation for effector protein secretion. A signal upon host cell contact that is transduced to the base of the secretion apparatus via subunits of the needle might be required to activate the secretion of effector proteins (310). A role of the needle subunits in the sensing and transduction of the activation signal was supported by the finding that individual point mutations in needle proteins from *Yersinia* spp. and *S. flexneri* lead to alterations in the secretion profile (Table 8).

Control of Effector Protein Export in *S. flexneri*

Control of effector protein secretion by an extracellular plug was also proposed for *S. flexneri*. Since the absence of the needle tip proteins results in constitutive secretion, it is assumed that the tip complex serves as an external plug that blocks secretion of effector proteins prior to host cell contact (569) (Tables 5 and 8). An additional protein involved in the control of effector protein secretion in *S. flexneri* is the secreted MxiC protein, which shares se-

quence and structural similarity with the YopN-TyeA complex from *Yersinia* spp. (132) and might act similarly to YopN. Notably, however, in contrast to the case for *yopN* mutants, which constitutively secrete translocon and effector proteins (176), deletion of *mxiC* leads to constitutive secretion of effector proteins but reduced secretion of translocon proteins in response to Congo red induction (350). This suggests that MxiC not only acts as a negative regulator of effector protein secretion but also promotes the secretion of translocon proteins. Interestingly, the formation of the tip complex is unaltered in the *mxiC* mutant. Since the *mxiC* mutant constitutively secretes effector proteins, it was proposed that the tip complex does not act as an external plug for effector protein export in the absence of MxiC (350).

Control of T3S in EPEC by SepL and SepD

In EPEC, T3S of translocon and effector proteins is controlled by SepL and SepD, which both localize to the bacterial membranes and interact with each other (137, 138, 408) (Table 5). Deletion of *sepL* leads to an increase in the secretion of effector proteins and to a reduced secretion of translocon proteins (137, 138). Since SepL shares homology with YopN and TyeA (see above) (426, 584), it might act together with SepD as a gatekeeper that promotes secretion of translocon proteins and prevents effector protein secretion prior to host cell contact. Interestingly, the C-terminal region of SepL interacts with the T3S chaperone CesL and the effector protein Tir but not with other effector proteins (584, 620). The SepL-Tir interaction appears to be required for the SepL-mediated control of effector protein secretion while being dispensable for the efficient secretion of translocon proteins (584). So far, the molecular mechanisms that link the SepL-Tir interaction to the control of effector protein secretion are not understood. However, given that Tir is the first effector protein that travels the T3S channel (see above), SepL-bound Tir might block the efficient access of additional effectors to components of the secretion apparatus, such as the ATPase EscN.

Control of Effector Protein Translocation by pH Sensing and a Sorting Platform in *Salmonella* spp.

As mentioned above, *Salmonella* spp. contain two translocation-associated T3S systems that are encoded by SPI-1 and SPI-2 and operate during bacterial entry into the host cell (SPI-1) and inside the host vacuole (SPI-2), respectively (Fig. 10). SPI-1- and SPI-2-mediated effector protein translocation is presumably controlled by the YopN homologs InvE (SPI-1) and SsaL (SPI-2). Deletion of *invE* and *ssaL* results in reduced secretion of translocon proteins and oversecretion of effector proteins, respectively, suggesting that InvE and SsaL not only act as negative regulators of effector secretion but also promote the secretion of translocon proteins (100, 289, 623). Notably, a similar phenotype was observed for *Salmonella* mutants lacking either SsaM or SpiC, two cytoplasmic regulatory proteins that interact with each other (621) (Table 5). SsaM and SpiC are involved in the control of SPI-2-mediated T3S, which is activated after acidification of the *Salmonella*-containing vacuole inside the host cell (450). It was previously shown that SsaM and SpiC bind to the YopN homolog SsaL at pH 5.0. The resulting SsaM-SpiC-SsaL complex promotes the secretion of translocon proteins and suppresses effector protein secretion. It might thus act as a gatekeeper for effector proteins when bacteria reside in the host vacuole (623). A shift to pH 7.2, however, leads to dissociation of the SsaL-SsaM-SpiC complex and to the activation of effector protein secretion. This pH shift might occur in nature when bacteria leave the host vacuole to enter the cytoplasm (623) (Fig. 10). The sensor for the pH shift is probably not the translocon, because a translocon mutant secretes wild-type levels of effector proteins at pH 7.2. It was therefore speculated that the extracellular pH is sensed by components of the needle (623). Alternatively, the tip complex could also serve as a sensor of the external pH. In this context, it is interesting that the tip complex protein SipD undergoes a conformational change at pH 5 to 6 (346). It remains to be investigated whether pH sensing is also involved in the control of effector protein secretion in other animal-pathogenic bacteria.

Control of translocon and effector protein secretion in *Salmonella* spp. not only depends on YopN-like gatekeeper proteins but also involves the predicted C ring of the T3S system. Experimental evidence suggests that the putative C ring provides a sorting platform for early, intermediate, and late substrates. Thus, the predicted C ring component SpaO was shown to associate with several proteins, including OrgA and -B (required for complex stability), the ATPase InvC, and translocon proteins (Table 2). Effector proteins are largely absent from the complex. However, they associate with SpaO-OrgA-OrgB in the absence of translocon proteins. Since translocon proteins are probably secreted prior to effector proteins, these findings suggest that effectors form a queue for docking to the predicted C ring (304). In the absence of the T3S4 protein InvJ (which leads to the constitutive secretion of needle and inner rod proteins but not of translocon proteins), translocon or effector proteins do not associate with the SpaO-OrgA-OrgB complex (304) (Fig. 10). It was therefore proposed that the selective binding of T3S substrates to the predicted C ring allows their sequential delivery. Interestingly, the binding of the SpaO-OrgA-OrgB complex to T3S substrates requires the presence of their cognate T3S chaperones, which might target their interaction partners to the predicted C ring (304). In conclusion, the published data on T3S in *Salmonella* spp. suggest that T3S is controlled not only by the gatekeeper proteins InvE and SsaL but also by SsaL-associated proteins that are released from SsaL upon a shift in the extracellular pH. Furthermore, the transport of effector proteins might also depend on the regulated binding of these proteins to the predicted C ring. Once again, this reveals the high complexity of T3S-associated control mechanisms.

CONCLUDING REMARKS

During the past 3 years, significant progress has been made in our understanding of the molecular mechanisms that underlie the assembly and control of T3S systems from Gram-negative pathogenic bacteria. While T3S systems have long been known as membrane-spanning nanomachines, the detailed characterization of several conserved components of T3S systems, including the analysis of protein crystal structures, has now shed more light on their contribution to substrate recognition and their function during the secretion process. Furthermore, recent studies revealed that the assembly of the T3S system occurs sequentially and probably involves two assembly platforms that are later joined together. The aims of future studies will certainly be to determine the location of every component of the T3S system and to generate a complete atomic model of the secretion apparatus.

In addition to the analysis of core constituents of the secretion apparatus, research on animal-pathogenic bacteria has also focused on the characterization of extracellular components of the

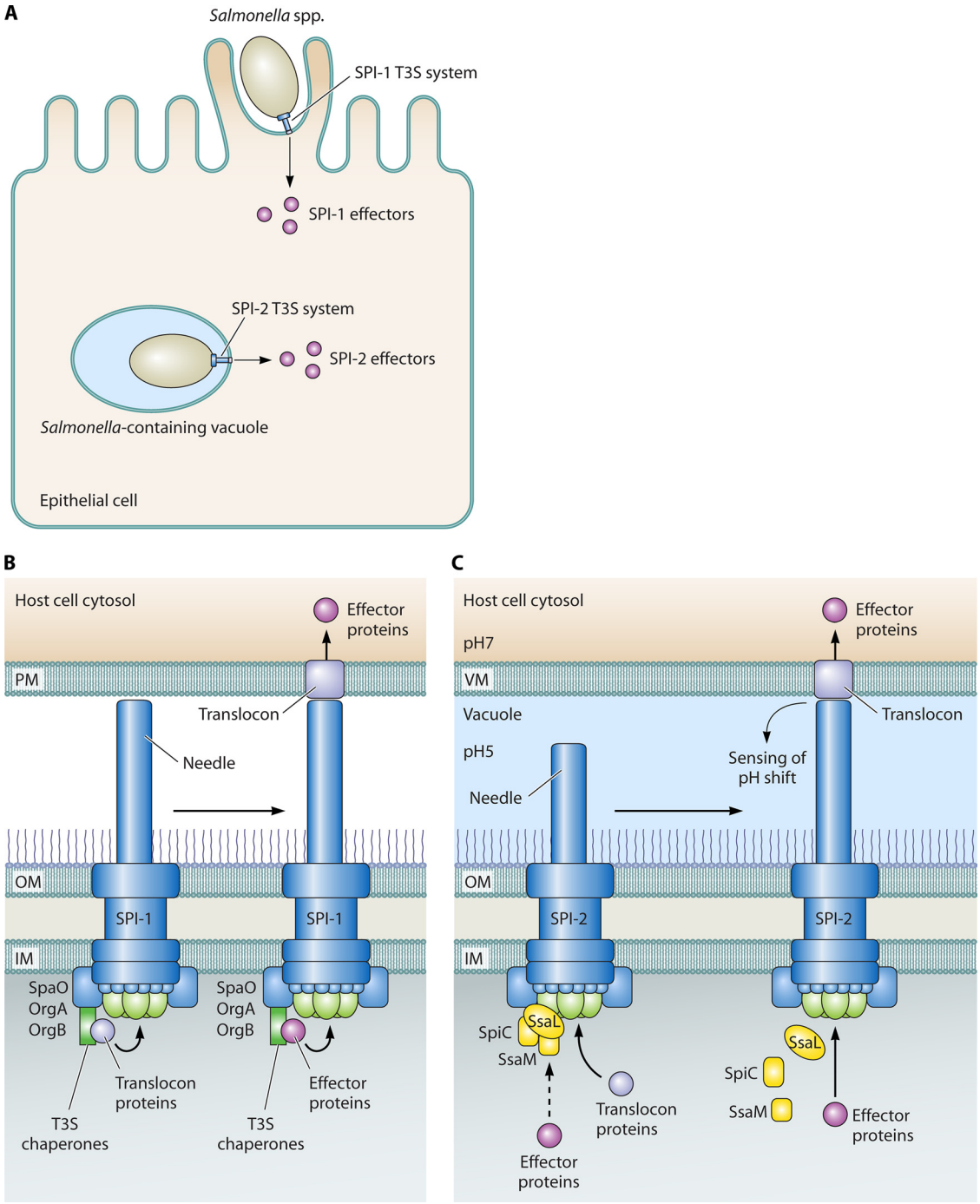


FIG 10 Control of SPI-1- and SPI-2-mediated T3S in *Salmonella* spp. (A) Infection of epithelial eukaryotic cells by *Salmonella* spp. The SPI-1-encoded T3S system injects effector proteins into epithelial cells, which leads to cytoskeletal rearrangements and membrane ruffling. Bacteria enter the host cell cytosol via endocytosis and activate the SPI-2-encoded T3S system inside the *Salmonella*-containing vacuole. (B) Predicted function of the SPI-1-encoded SpaO-OrgA-OrgB complex during control of T3S. The SpaO-OrgA-OrgB complex serves as a docking platform for translocon and effector proteins that are sequentially targeted to this complex by a process that presumably requires the presence of corresponding T3S chaperones. According to the predicted hierarchy in T3S, effector proteins form a queue for docking to the SpaO-OrgA-OrgB complex, while translocon proteins are secreted. (C) Control of SPI-2-dependent secretion of translocon and effector proteins. A complex of SpiC, SsaL, and SsaM controls the secretion of translocon and effector proteins dependent on differences in the external pH. At pH 5 (inside the *Salmonella*-containing vacuole), the complex blocks the efficient secretion of effector proteins, while translocon proteins are secreted. A shift in the extracellular pH to pH 7 leads to the dissociation of the SpiC-SsaL-SsaM complex and thus induces effector protein secretion. The pH shift is probably sensed by the extracellular components of the T3S system. Note that the architecture of the SPI-2 T3S system is speculative and is proposed according to amino acid sequence similarities between predicted components of SPI-2 T3S systems and known translocation-associated T3S systems. The dashed arrow indicates a reduced secretion and/or translocation rate.

T3S system, such as needle, translocon, and tip proteins. Experimental evidence suggests that these proteins are involved in the sensing and transduction of external signals such as the pH or host cell contact. In contrast, not much is known about the identities and functions of translocon and potential tip complex proteins from plant-pathogenic bacteria. Future research should help to functionally characterize these proteins, because it cannot be assumed that the contributions of individual proteins to the assembly and activity of the T3S system are similar in different pathogens. We also still know very little about the functions of the nonconserved components of T3S systems from plant- and animal-pathogenic bacteria. Since these proteins might reflect adaptions of the T3S system to different host organisms or extracellular environments, they should be included in future studies.

Given the architecture of T3S systems, it has been assumed that T3S is a hierarchical process. Recently, various regulatory proteins of T3S systems that are involved in transcriptional and posttranscriptional control mechanisms or the switch in substrate specificity have been characterized intensively. However, the precise mechanisms that lead to the activation of T3S and guarantee the hierarchical secretion of early, middle, and late T3S substrates are far from being understood and so far have been studied mainly in animal-pathogenic bacteria. Future research should therefore focus on the detailed characterization of individual T3S control proteins and on the analysis of their interactions with substrates and components of the T3S system. Since T3S control proteins and substrate recognition sites are not highly conserved and the experimental findings reported to date have already revealed genus- and species-specific differences in the control mechanisms underlying T3S, the characterization of individual species- and even pathovar-specific proteins will be crucial for a complete understanding of the complex T3S-associated regulatory mechanisms. Furthermore, this knowledge will help in the design of inhibitors of T3S that may be used as therapeutic agents and in development of biotechnological approaches that will employ the T3S system as a tool for the targeted and controlled delivery of foreign proteins into eukaryotic cells.

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